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Immune activation of sulfated polysaccharide isolated from *Ecklonia cava* and its biological mechanism

Ginnae Ahn

Department of Marine Life Science

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Gin Nae Ahn
(Supervised by Professor Ki-Wan Lee)

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This thesis has been examined and approved.

Thesis director, You-Jin Jeon, Prof. of Marine Life Science

Youngheun Jee, Prof. of Veterinary Medicine

Ki-Wan Lee, Prof. of Marine Life Science

Ki Young Kim, Prof. of Marine Life Science

BuHyun Youn, Prof. of Biological Sciences, Pusan National University

Date 2010. 08

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CONTENTS

국문초록

Polysaccharide(다당류)는 단당류의 복합체로서, 육상식물과 해양식물 등으로부터 추출되어 항응고, 항염증, 면역조절, 면역활성과 같은 다양한 생리활성을 가진다고 보고되어왔다. 특히, 다당류는 면역조절이나 면역활성과 같은 면역반응에 있어 뛰어난 효능을 보인다고 보고되었고, 그에 따라, 많은 연구자들에 의해 주목 받아왔다. 뿐만 아니라, 최근 들어서는 해조류로부터 다당류를 분리하고 그들이 면역반응에 미치는 영향에 대해 많은 연구가 진행되고 있다. 또한, 이전 연구에서 다당류를 함유하고 있는 감태 (*Ecklonia cava*)가 면역조절, 면역활성 및 항암, 항산화 활성과 같은 다양한 생리활성을 가진다고 보고되었다. 따라서, 이번 연구에서는 갈조류의 한 종류인 다시마목 다시마과에 속하는 감태로부터 다당류를 분리하고, 분리된 다당류의 면역활성 효과와 그 기전을 밝히고자 하였다. 이 연구는 크게 세 개의 부로 나누어졌으며, 제 1부(PART 1)에서는 감태로부터 분리된 다당류가 마우스 비장세포의 활성화, 증식과 분화 등에 대해 미치는 효과와 그것의 기전을 확인하였고, 제 2부(PART 2)에서는 방사선 조사된 마우스 비장세포에 대한 감태로부터 분리된 다당류의 방호효과를 확인하였다. 또한, 제 3부(PART 3)에서는 방사선 조사에 의해 유도된 마우스 면역억제모델에서 면역기관이나 조혈기관 등에서 감태로부터 분리된 다당류의 방호기전을 확인하였다.

제 1부(PART 1)에서는 감태로부터 분리된 다당류가 약 72.25%의 fucose 함량과 약 6.17%의 황산기를 함유하고 있는 황산 다당류(sulfated polysaccharide, SP)라는 것을 확인할 수 있었다. 또한, 마우스 비장세포에 SP를 처리하였을 때, 아무것도 처리하지 않은 세포군보다 세포 독성 없이 세포의 증식을 상당히 유의성 있게 증가시킨 것을 확인할 수 있었고, SP 처리 후, 24 시간째 보인 세포의 증식 효과는 다른 시간대들에 비해 상당히 높았다. 또한, 증식된 비장세포 내에서, 특정 세포 타입을 확인한 결과, $CD3^+$ mature T cells, $CD45R/B220^+$ pan B cells, $CD11b^+$ macrophages와 $Gr\text{-}1^+$ granulocytes의 증식과 분화가 SP

처리에 의해 상당히 증가되었다. 게다가, SP 처리는 T 세포의 초기 자극 인자로 알려진 사이토카인 IL-2의 mRNA 발현 및 단백질 분비를 상당히 증가시켰다. 또한, B lymphocytes에서 생성되는 항체들로 알려진 immunoglobulin(Ig)G1과 IgG2a에 대한 SP의 효과를 확인한 결과에서, SP의 처리가 IgG1과 IgG2a의 생성을 약간 증가시켰다는 것을 제시했다. 그리고, T와 B lymphocytes의 활성화와 증식, 분화를 이끄는 역할을 한다고 알려진 mitogen-activated protein kinases(MAPKs)와 nuclear factor kappa B(NFκB) signal pathway가 SP에 의해 증가된 면역세포의 증식과 IL-2 생성에 있어 영향을 미치는 지를 확인하였다. 그 결과, JNK, serine protease와 NFκB의 억제자로 알려진 SP600125, TPCK, PDTC를 처리한 경우, SP가 유도한 비장세포의 증식과 IL-2 사이토카인의 생성이 상당히 유의성 있게 억제되었다. 또한, SP의 처리는 인산화된 JNK, IκB와 핵 내 c-fos와 NFκB의 발현을 상당히 증가시켰으며, AP-1-과 NFκB-DNA binding을 활성화 시켰다. 그러나, SP600125, TPCK, PDTC를 처리한 경우, SP에 의해 유도된 JNK와 NFκB의 활성화는 상당히 억제되었다. 이 결과로부터, SP가 JNK/NFκB signal pathway를 활성화시켜 마우스 비장세포에서 IL-2의 발현과 생성을 유도함으로써 비장세포의 활성화, 증식과 분화를 유도하였다는 것을 확인하였다.

제 2부(PART 2)에서는 방사선 조사가 유도한 세포의 손상으로부터 SP가 마우스 비장 세포를 보호할 수 있는 지를 확인하였다. 그 결과, SP를 처리함에 따라 방사선 조사에 의해 감소된 비장 세포의 생존과 증식이 농도의존적으로 상당히 증가하였다. 또한, 방사선 조사에 의해 증가된 ROS의 생성이 SP 처리에 의해 유의성 있게 감소한 것을 확인하였다. 그리고, SP는 방사선이 유도한 tail DNA percentage(%), olive tail DNA movement(μm), sub-G1 apoptotic DNA(%)에 따른 DNA 손상을 상당히 억제시켰다. 게다가, SP는 방사선 조사된 비장세포에서 apoptosis 유발 단백질인 Bax의 발현을 감소시키고, apoptosis 억제 단백질인 Bcl-2와 Bcl-xL의 발현을 증가시킴으로써 세포의 apoptosis를 억제시켰다. 이 결과는 SP가 방사선 조사된 세포에서 Bax와 Bcl-2, Bcl-xL과 같은

apoptosis 관련 단백질의 발현을 조절하고, ROS의 생성을 감소시킴으로써 DNA 손상을 억제시켜 보호효과를 유도한다는 것을 제시하였다.

마지막으로, 제 3부(PART 3)는 방사선 조사에 의해 억제된 마우스 면역 억제 모델에서 면역기관이나 조혈기관 등에서 감태로부터 분리된 다당류의 방호기전을 확인하였다. 먼저, SP가 독성이 없는 지를 확인하기 위해, 단회 독성 시험(2000 and 5000 mg/kg B.W., 첫날은 6시간 동안 한 시간마다 경구투여, 그 뒤부터 14일 간 매일 1회 경구투여)과 아급성 독성 시험(1, 10, 100 mg/kg B.W., 매일 1회 21일 간 경구투여)을 각각 수행하였고, 그 결과, SP 투여 후, 죽은 마우스가 관찰되지 않았고, 체중과 심장, 신장, 간, 비장, 폐와 같은 조직의 무게와 혈액세포 수에 대한 변화도 관찰되지 않았다. 이것으로부터 SP가 동물에 투여되었을 때, 독성을 가지지 않는 다는 것을 확인하였다. 다음으로 방사선이 유도한 세포의 DNA 손상에 대한 SP의 효과를 확인한 결과에서, 방사선 조사가 유도한 peripheral blood lymphocytes와 splenocyets의 tail DNA percentage(%), olive tail DNA movement(μm), tail DNA length(μm)의 증가와 같은 DNA 손상이 SP 투여에 의해 상당히 감소되었다. 방사선 조사에 의해 감소된 마우스 비장세포의 증식은 SP 투여에 의해 유의성 있게 상당히 증가되었다. 또한, SP의 hematopoietic 능력을 확인한 결과에서, SP 투여는 방사선 조사 대조군에 비해 비장 크기와 체중에 대한 비장의 무게, colony forming units(CFU)의 수를 상당히 증가시켰고, bone marrow cells의 수도 유의성 있게 증가시켰다. 게다가, 방사선 조사에 의해 감소된 소화 면역기관인 소장 내 villi의 길이와 crypt의 수, crypt 내 apoptotic fragment의 수가 SP 투여에 의해 상당히 유의성 있게 증가되었다. 또한, 방사선 조사에 의해 증가된 마우스 소장 내 apoptosis 유발 단백질인 P53과 Bax의 발현은 SP 투여에 의해 감소되었고, 감소된 apoptosis 억제 단백질인 Bcl-2의 발현은 SP 투여에 의해 증가되었다. 흥미롭게도, SP는 방사선 조사에 의해 융모 내 점막고유층 세포에서 증가된 p53의 발현과 융모 상피세포에서 증가된 Bax의 발현을 감소시켰다. 그에 반해, 방사선 조사에 의해 감소된 융모 내 점막고유층 세포와 소장움와

세포에서 Bcl-2의 발현을 SP 투여는 상당히 증가시켰다. 그 결과, SP는 조혈기관(장, 골수)과 말초면역기관(혈액, 비장)에 존재하는 면역세포의 손상을 감소시키고, apoptosis 연관 단백질인 P53과 Bax, Bcl-2과 같은 단백질 발현을 조절함으로서 마우스를 산화적 스트레스로부터 보호한다는 것을 확인하였다.

이와 같은 결과를 종합해볼 때, 감태로부터 분리된 SP는 MAPKs과 NFκB signal pathway를 활성화시켜 면역세포의 활성화를 이끌고, 그것은 산화적 스트레스가 유도한 면역 억제 모델에서 손상된 면역 세포와 조직의 기능을 회복시킴으로써 면역활성 효능을 가짐을 알 수 있었다.

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identified by RT- PCR. (B, C) The population of $CD3^+$-mature T lymphocytes secreting IL-2 and production of IL-2 in SP-treated splenocytes were measured by intracellular cytokine staining assay and ELISA. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$.

Figure 1-7. Effects of SP treatment on IgG1 (A) and IgG2a (B) in splenocytes. Splenocytes were treated or not with SP (150 μg/ml) for 72 h and the supernatants were used to measure the concentrations of IgG1 and IgG2a using commercially available ELISA kits. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$.

Figure 1-8. Blockage by MAPK inhibitors including JNK, p38, and ERK on the proliferation (A) and total IL-2 production (B) of splenocytes induced by SP. (A) After incubation with SP for 24 h, the supernatants were used for ELISA analysis. (B) The splenocyte's proliferation 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.E. *, $p < 0.05$, ***, $p < 0.005$.

Figure 1-9. Effects of SP on expressions of cytoplasmic pan-JNK and phospho-JNK (A), nuclear c-fos (B), and nuclear AP-1 DNA binding activity (C). The cytoplasmic and nuclear proteins were extracted from splenocytes incubated with SP (150 μg/ml) for 0.25, 0.5, 1, 6, 12, and 24 h. The cytoplasmic (50 μg) (A) and nuclear (40 μg) (B) proteins were immunoblotted with pan-JNK, phosphor-JNK, c-fos, and β-actin antibodies. (C) Nuclear proteins (10 μg) were assayed for Ap-1 activation as described in Materials and Methods.

Figure 1-10. Blockage by SP600125, a JNK inhibitor on expressions of cytoplasmic phospho-JNK (A), nuclear c-fos (B), and nuclear AP-1 DNA binding activity (C). The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with SP (150 μg/ml) and SP600125 (20 μM) for 0.5 h. The cytoplasmic (50 μg) (A) and nuclear (40 μg) (B) proteins were immunoblotted with phospho-JNK, c-fos, and β-actin antibodies. (C) Nuclear proteins (10 μg) were assayed for Ap-1 activation as described in Materials and

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INTRODUCTION

Marine algae that can be divided into three basic types: brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta) seaweed have been reported to contain diverse classes of biologically active compounds which are useful in the pharmaceutical industry. In addition, they contain large amount of polysaccharides, such as alginate, fucoidan, laminaran, agarose, and carrageenan and polyphenols such as phloroglucinol, eckol, and dieckol. Especially, *Ecklonia cava* (*E. cava*) used in this study is a species of brown seaweed found in the ocean of Japan and Korea and contains the polyphenols, polysaccharides, fucoidan, alginate, fucan, and laminarin, which are water-soluble dietary fibers, and phycocolloids. *E. cava* have showed the various biological effects such as anti-oxidant *in vitro,* anticancer, anticoagulant, immunomodulation, anti-inflammation, immune activation and matrix metalloproteinase inhibition effects. Particularly, its polyphenol compounds such as eckol, dieckol, phloroglucinol, and triphlorethol-A have showed various biological effects such as anti-oxidant, anticancer, anticoagulant, immunomodulation, anti-inflammation, immune activation and matrix metalloproteinase inhibition effects for few decades. So, it is widely available as an herbal remedy in the form of an extract called Seanol, a polyphenol complex that, according to the manufacturer, is an extremely potent antioxidant compared to any and all land-based peers. However, there are little the studies about the biological effects of

polysaccharides isolated from *E. cava*, although they showed anticoagulant and anticancer effects. Therefore, this study wanted to additionally reveal the biological effects of the polysaccharide from *E. cava*.

Normally, immune system such as the lymphoid and hemopoietic systems processes within an organism that protects against disease by identifying and killing pathogens and tumor cells. It detects a wide variety of agents such as pathogens and virus and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly. Recently, in developed countries, obesity, alcoholism, malnutrition and drug use are common causes of poor immune function such as immunodeficiency. In addition, in many countries, many people die from cancer which causes immunosuppression every year and the number is gradually increasing. So, enhancing immune systems inhibited in radio-therapy of cancer patients are important and remains an unsolved problem. At these points, to enhance the capacities of immune system including immune cells such as lymphocytes, monocytes, granulocytes, stem cells and so on, many researchers have been trying for few years. Here, Part 1 of this study also investigated the biological capacities of the polysaccharide isolated from *E. cava* (SP) on the enhancement such as the activation, proliferation, and differentiation of splenocytes including lymphocytes, monocytes, and granulocytes known as representative immune cells in immune systems. Additionally, in representative immune

suppression animal model, this study wanted to reveal the capacities of polysaccharide on enhancement of immune system including the lymphoid and hemopoietic systems. So, in Part 2 and 3 of the present study, exposure of gamma ray irradiation was used as a tool to cause the destruction of the lymphoid and hemopoietic systems via increasing apoptosis of not only proliferating stem cells including bone marrow and intestinal crypt cells, but also splenocytes including lymphocytes. Therefore, Part 2 investigated whether SP has radio protective effects as enhancing the survival and proliferation of murine splenocytes as well as inhibiting apoptosis by modulating apoptotic protein expressions. Part 3 identified whether SP leads to radio protective effects via enhancing immunostimulatory and hematopoietic capacities and inhibiting DNA damages by apoptosis in mice exposed to a lethal dose of gamma ray irradiation without toxicities.

PART 1

The JNK/NFκB pathway is required for activation of murine splenocytes induced by sulfated polysaccharide isolated from *Ecklonia cava*

Abstract

Background: Polysaccharide has been used as a material in functional food and medicine industry due to its capacities on antioxidant, immunomodulation and immune activation. Previous studies have reported that *Ecklonia cava* (Alariaceae, Phaeophyta) containing plentiful polysaccharide has the beneficial effects for antioxidation, immunomodulation and immune activation. This study investigated whether sulfated polysaccharide (SP) of *E. cava* has immunological response and its molecular mechanisms, specifically focusing on the functional activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NFκB) involved in the activation, proliferation and differentiation of murine splenocytes

Methods: First, to investigate whether SP leads to the proliferation and differentiation of murine splenocytes without cytotoxicity, MTT assay, ^{3}H-thymidine incorporation, flow cytometry, and 5-,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) assay were performed. Then, to assess the functional capacities of SP, the mRNA expression and/or production of cytokine such as interleukin (IL)-2, an important key cytokine stimulating T lymphocytes and immunoglobulin (Ig) G1 and IgG2a were examined by real time-polymerase chain reaction (PCR), intracellular cytokine assays, and/or enzyme-linked

immunosorbent assay (ELISA). In further, SP's molecular mechanisms were assessed by Western blot, and electrophoretic mobility shift assay (EMSA).

Results: SP markedly enhanced the proliferation and differentiation of splenocytes including $CD3^+$ mature T cells, $CD45R/B220^+$ pan B cells, $CD11b^+$ macrophages, and $Gr\text{-}1^+$ granulocytes without cytotoxicity. In addition, mRNA expressions and productions of IL-2 were markedly increased by SP treatment, compared to those in non-treated cells (control cells). Also, SP slightly increased the production of IgG1 and IgG2a, compared to control cells. Interestingly, the proliferation and IL-2 production of splenocytes stimulated by SP were significantly inhibited the application of JNK (SP600125) or NFκB (TPCK and PDTC). Also, SP indeed induced the phosphorylation of JNK and IκB and the activation of nuclear c-fos and NFκB p65 as well as Ap-1 and NFκB p65 DNA binding, as compared to control cells. Moreover, the activations of JNK and NFκB pathway induced by SP were markedly blocked by the application of JNK and NFκB inhibitors.

Conclusion: These results suggest that SP induced the activation, proliferation and differentiation of splenocytes as stimulating IL-2 production through the JNK/NFκB pathway.

1-1. Introduction

Algal polysaccharide including agar, carrageenans, alginates, laminaran, rhamnan sulfate, and fucoidan isolated from seaweeds are known as the popular materials for the food, agriculture and other related industries (Athukorala et al., 2009). At these points, during the last few decades, many researchers have focused on the isolation of polysaccharides and their beneficial biological effects. Indeed, the biological functions of algal polysaccharides have been previously characterized, including immunomodulatory, anti-inflammatory, anticoagulant, anticancer, antiadhesive and antiabgiogenic activities (Matsuda et al., 2003; Athukorala et al., 2006 and 2009; Teruya et al., 2007; Cumashi et al., 2007; Guzmán et al., 2003). Especially, many researchers have shown interest in the immunological capacities of polysaccharides and reported that they play impotent roles as immunomodulator or immune activator (Sun et al., 2009; Naira et al., 2004; Zhua et al., 2007). In recently, the previous studies have also demonstrated that *Ecklonia cava* (*E. cava*) containing plentiful polysaccharide induced beneficial effects on immunomodulation, anti-inflammation, immune activation (Athukorala et al., 2009; Ahn et al., 2008a,b). However, analyzing immunological capacities of polysaccharides of *E. cava* and its mechanism has not yet revealed.

Normally, the transmission of extracellular signals to intracellular targets for the

activation, differentiation, and proliferation of immune cells is mediated by several proteins such as mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases (ERK1/2), Jun-NH_2-terinal kinases (JNK), and p38, and nuclear factor-κB (NFκB) (Garaude et al., 2005 and 2006; O'Reilly et al., 2009; Gerondakis et al., 1998; Liu et al., 2004). Indeed, previous studies have indicated that MAPKs and NFκB pathways led to immune responses by promoting the transcription of essential target genes in T and B lymphocytes and monocytes (Gerondakis et al., 1998; Carretta et al., 2009; Pimentel-Muiños et al., 1994; Armitage RJ et al., 1995; Varga et al., 1999; Yamamoto et al., 2001; Prasad et al., 2002; Granucci et al., 2003; Mora et al., 2003; Dumont et al., 1998). Moreover, they play as important role for encoding and synthesizing cytokine such as IL-2, which in turn stimulates the activation, proliferation, and differentiation of T lymphocytes as well as the expression of IL-2 receptors (Karin et al., 2000; Pahl et al., 1999; Mora et al., 2003; Pimentel-Muiños et al., 1994; Yamamoto et al., 2001; Prasad et al., 2002). At these points, clearly identifying whether the immunological capacities of polysaccharide of *E. cava* are related with the MAPKs and NFκB pathways remains.

Here, this present study demonstrates that a polysaccharide of *E. cava* stimulates the survival, proliferation and differentiation of splenocytes through the MAPKs and/or NFκB pathway, thereby augmenting the expression and production of IL-2.

1-2. Materials and Methods

1-2-1. Preparation of enzymatic extract from *E. cava*

E. cava collected from the coast of Jeju Island, South Korea was washed with fresh water, freeze-dried and pulverized into powder with a grinder. Ten gram of the dried *E. cava* was homogenized with 1 L of distilled water (pH 6.0) and mixed with 1 mg of AMG (Novo Nordisk, Bagsvaerd, Denmark). The reaction was conducted at 40°C for 12 h and the digest was boiled for 10 min at 100°C to inactivate the enzyme. The product was clarified by 20 min of centrifugation at 3000 × *g* to remove any unhydrolyzed residue. After filtration of the enzymatic extract, the supernatant was adjusted to pH 7.0, freeze-dried and then stored for use in further experiments.

1-2-2. Isolation of polysaccharides from *E. cava* (SP) and its composition

To isolate a polysaccharide from the enzymatic extract, ethanol (2 L) was added into the enzymatic extract (1 L) and kept in 4℃ for 24 h. After centrifugation, a polysaccharide was isolated from the precipitation and freeze-dried for 3 days. Then, the composition of the polysaccharide was analyzed by measuring the contents of sulfate group and monosaccharides such as fucose, xylose, galactose, mannose, and glucose known as the standard markers of various polysaccharides according to a modified version of methods

indicated in the previous study (Saito et al., 1968; Ahn et al., 2008).

1-2-3. Isolation of splenocytes from spleen

Spleens were removed aseptically from ICR mice, ages 6 to 9 weeks (SLC, Inc., Shizuoka, Japan), and red blood cells were lysed by immersion in ACK lysis buffer including 0.84% ammonium chloride at room temperature for 10 min in darkness. After washing with Dulbecco's phosphate-buffered saline (DPBS) (Gibco BRL, Life Technologies, New York, USA) containing 1% fetal bovine serum (FBS), the purified splenocytes were suspended in (Roswel Park Memorial Institute) RPMI medium containing 10% FBS and 1% antibiotic (100 U/ml penicillin and 100 mg/ml streptomycin) (Sigma, St. Louis, MO, USA) to use directly for experiments.

1-2-4. MTT assay

The viability of splenocytes exposed to SP was checked by an MTT assay known as a colorimetric assay that is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mossmann, 1983). The splenocytes (1×10^5 cells) were incubated with SP at various concentrations (from 25 to 150 μg/ml) for 24, 48, and 72 h. Control cells were treated with

only RPMI medium. Then, MTT stock solution (10 μl; 5 mg/ml) was applied to each of the wells for 4 h. The absorbance of formazan crystals dissolved in 100 μl of solublization buffer including 50% DMSO and 20% sodium dodecyl sulfate (SDS) (pH 8.4) was measured at 540 nm. The optical density of the formazan generated in control cells was considered to represent 100% viability. The data were expressed as mean percentages of the viable cells versus the respective control.

1-2-5. ^{3}H-thymidine incorporation assay

Generally, ^{3}H-thymidine incorporation assay is a 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, USA). This assay was performed in order to ascertain the proliferation of splenocytes exposed to SP. The cells (4×10^{5}) were seeded in 96 well culture plates (Nunc, Copenhagen, Denmark) and incubated with SP (25, 50, or 150 μg/ml) and/or SB203580 (20 μM) which is a P38 inhibitor, SP600125 (20 μM) which is a JNK inhibitor, PD98059 (20 μM) which is a ERK inhibitor, TPCK (tosyl phenylalanyl chloromethyl ketone, 20 μM) which is a serine protease inhibitor, or PDTC (pyrrolidine dithiocarbamate, 750 nM) which is a NFκB inhibitor, respectively. Concanavalin A (Con A) (2 μg/ml) was used for positive control cells. After incubation for 24, 48, or 72 h at 37°C,

95% humidity and 5% CO_2, 1 μCi of ^{3}H-thymidine (specific activity 42 Ci/mmol) was added to each well, and the plates were incubated for an 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.

1-2-6. Flow cytometry assay

To detect the specific cell types in splenocytes proliferated by SP, the flow cytometry assay, which simultaneously measures multiple characteristics of single cells at a rapid rate, was used in this experiment. Briefly, the splenocytes (5×10^6 cells) were cultured with 150 μg/ml of SP in 24 well culture plate (Nunc, Copenhagen, Denmark). After 24 h, the splenocytes were washed with DPBS and blocked with an anti-mouse immunoglobulin (Ig) G solution in DPBS for 15 min at 4°C to inhibit nonspecific staining. Then, the cells were stained with fluorescently labeled monoclonal antibodies (mAbs) to the mouse antigens of interest at optimal concentrations for an additional 15 min at 4°C. Abs (BD Biosciences, San Jose, CA, USA) were directly labeled with the following fluorescent tags: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- for CD3 (145-2C11 and American Hamster IgG1*,κ), CD45R/B220 (RA3-6B2 and rat $IgG2_a$,κ), CD11b (M1/70 and rat(DA) IgG_{2b},κ), and Gr-1 (RB6-8C5 and Rat IgG2b,κ). Those used in these experiments were CD3-FITC as a

specific marker for mature T lymphocytes, CD45R/B220-FITC as a specific marker for pan B lymphocytes and abnormal T cells, CD11b as a specific marker for granulocytes and macrophages, and Gr-1 as a specific marker for granulocytes. Appropriate isotype controls were always included. After centrifugation, the cells were fixed with 1% formalin, and 20,000 viable cells per treatment (as determined by light scatter profiles) were analyzed using a BD FACSCalibur^{TM} flow cytometer and CellQuest software (BD Biosciences). Also, the population proliferated lymphocytes, monocytes, and granulocytes were distinguished by multi-parameter flow cytometry.

1-2-7. Carboxy fluorescein diacetate succinimidyl ester (CFSE) assay

To identify the effects of SP on the division of splenocytes, CFSE assay was performed. Normally, after cell division, CFSE labeling is distributed equally between daughter cells, each of which is, therefore, half as fluorescent as the parent. For this assay, the splenocytes (6×10^7 cells) were suspended in warm RPMI without FBS and mixed with a working solution of 50 μM 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 CFSE dye. The CFSE-labeled cells were washed in cold RPMI and resuspended in RPMI 1640 medium containing 10% FBS and 1% antibiotic. Then, 3×10^6 CFSE-labeled cells were

incubated with 150 μg/m of SP or vehicle at 37°C for 5 days, and the medium was replaced with fresh RPMI medium containing 10% FBS and 1% antibiotic at 3 days. After 5 days, the CFSE-labeled cells were harvested and stained with CD3, CD45R/B220, CD11b, and Gr-1 mAbs-PE. Data was collected for 50,000 cells and analyzed using a BD FACSCalibur™ flow cytometer and CellQuest software (BD Biosciences).

1-2-8. Real time polymerase chain reaction (RT-PCR)

Real time PCR analysis was used to identify the effects of SP on mRNA expression level of IL-2 cytokine. Splenocytes were incubated with SP (150 μg/ml) for 6, 12, and 24 h before RNA extraction. After incubation, the cells were harvested and lysed in the Trizol reagent (Molecular Research Center, Inc., Cincinnati, Ohio, USA). The addition of chloroform (Sigma) and incubation for 5 min at 4°C followed. Supernatants obtained after centrifugation were mixed with isopropanol (Sigma), and the resulting RNA pellets were washed with 80% EtOH and stored at -20°C until use.

The cDNA was synthesized with RNA purified from the splenocytes by using a Promega A3500 kit, according to manufacturer's instructions (Promega, San Luis Obispo, CA, USA). PCR of this cDNA and the primer (Bioneer, Daejeon, South Korea) displayed in Table 1-1 was performed for 50 cycles with a 5 min denaturing step at 95°C, a one min

annealing step at 53 to 60°C and a 2 min extension phase at 72°C using the TaKaRa real time PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were run on a 1.5% EtBr/agarose gel and visualized by UV transillumination.

1-2-9. Intracellular cytokine staining assay

To investigate the effects of SP on the cells secreting interleukin (IL)-2, intracellular cytokine staining assay was used in this experiment. Briefly, 3×10^6 cells were incubated with 150 μg/ml of SP or vehicle at 37°C for 24 h and re-incubated with a cocktail containing brefeldin-A, ionomycin and phorbol 12-myristate 13-acetate (PMA) for intracellular cytokine staining. After 5 h of stimulation, the cells were harvested and washed with DPBS. The cells were blocked with anti-mouse IgG solution in DPBS for 15 min at 4°C to inhibit nonspecific staining, and then stained with fluorescently labeled CD3-FITC at optimal concentration for an additional 15 min at 4°C. Then, the cells were reacted with the IL-2 mAb-PE (BD Biosciences). After the reaction, the cells were washed and fixed with 1% formalin. Twenty thousand viable cells per treatment (as determined by light scatter profiles) were analyzed using a BD FACSCalibur™ flow cytometer and CellQuest software.

Table 1-1. The sequence of primer used in this study

Primer	Primer 5'–3' sequence (F: forward; R; reverse)	RefSeq ID
IL-2	F: CCTGAGCAGGATGGAGAATTACA R: TCCAGAACATGCCGCAGAG	NM 008366

1-2-10. Enzyme linked immune absorbent assay (ELISA)

ELISA was performed to identify the effects of SP on the productions of cytokines such as IL-2 and IgGs such as IgG1a and IgG2b. For this assay, the splenocytes were incubated with SP (150 μg/ml) and/or SP600125 (20 μM), TPCK (20 μM), or PDTC (750 nM) for 24 and/or 72 h, and the supernatants were collected from the cell suspensions. Then, these supernatants were analyzed according to the manufacturer's recommendations with mouse cytokine-specific ELISA kits (Biosource International, Camarillo, CA, USA) for the production of cytokines and ELISA Starter Accessory Kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) and Bethyl Quantitation ELISA sets (Bethyl Laboratories, Inc.) for the production of immunoglobulins. Cytokine and immunoglobulin levels were calculated with standard curves using recombinant murine cytokines or immunoglobulin.

1-2-11. Western blot analysis

Western blot analysis was performed to assess whether SP is related to the MAPK and/or nuclear factor κB (NFκB) pathway in splenocytes. Cytoplasmic (50 μg/well) and nuclear protein (40 μg/well) preparations were loaded into sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrophoresed under denaturing conditions.

Subsequently, proteins were electro-transferred onto nitrocellulose transfer membrane. After blocking with 5% nonfat milk for 2 h, blots were incubated with JNK (1:1000 dilution), phospho-JNK (1:1000 dilution), c-fos (1:1000 dilution), phospho-IκBα (1:1000 dilution), IκBα (1:1000 dilution), NFκB p65 (1:1000 dilution), or β-actin (1:3000 dilution) antibodies for 60 min followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG for 60 min. Visualization was achieved by using ECL reagents.

1-2-12. Electrophoresis mobility shift assay (EMSA)

EMSA was used to identify whether SP leads to Ap-1 and/or NFκB p65 DNA binding activities in splenocytes. Ap-1 and NFκB p65 DNA binding oligonucleotides were 3′-biotinylated and annealed using the biotin 3′-end DNA labeling kit (Pierce, Rockford, IL, USA). The 20 fmol of biotin-end-labeled target DNA and 10 μg of nuclear proteins were used for binding reactions using LightShift™ chemiluminescent EMSA kit (Pierce). The binding mixtures were loaded onto 4% polyacrylamide gels and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond™-N^+) in 0.5 × Tris borate/EDTA at 380 mA for 1 h. Transferred DNA samples were cross-linked and detected using HRP-conjugated streptavidin (LightShift™ chemiluminescent EMSA kit) according to the manufacturer's instructions.

1-2-13. Statistical analysis

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

1-3. Results

1-3-1. Polysaccharide of *E. cava* contained the plentiful contents of fucose and sulfate group

The components of SP isolated from enzymatic extract of *E. cava* were quantified by analyze its monosaccharide and sulfated group contents. As expressed in Figure 1-1 and Table 1-2, the crude polysaccharide isolated from enzymatic extract of *E. cava* were consisted mainly of fucose (about 72.25%) and sulfate group (about 6.17%) with small quantities of other monosacchardies such as galactose (about 12.28%), xylose (about 8.04%), mannose (about 4.74%) and glucose (about 1.82%). These results indicate that SP can be regarded as the sulfated polysaccharide known as a fucoidan due to its high fucose and sulfate group contents.

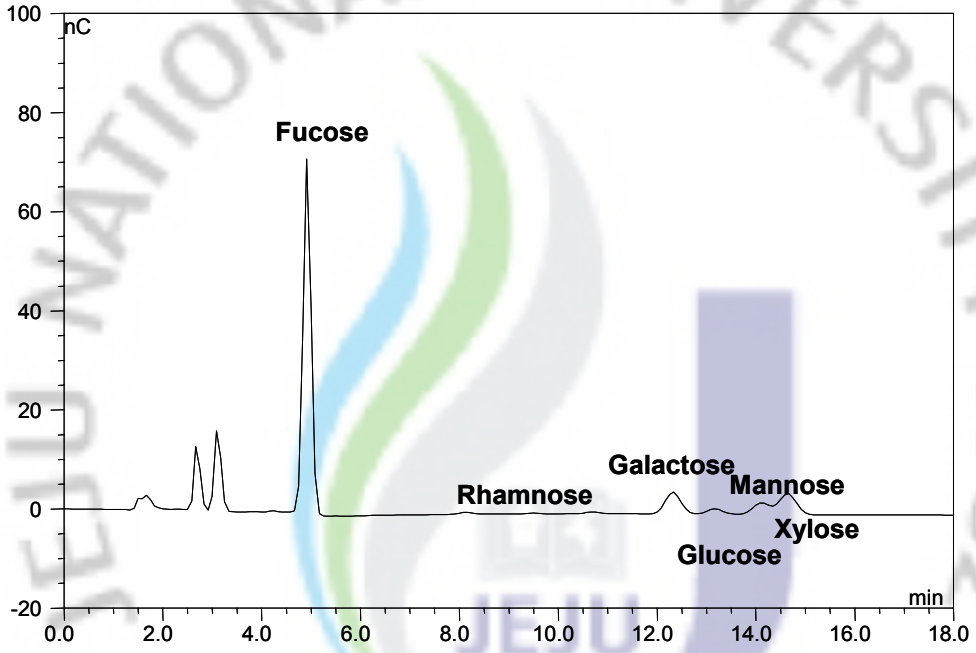

Figure 1-1. Chromatogram on monosaccharide contents of a polysaccharide isolated from *E. cava*

Table 1-2. The monosaccharide and sulfate group composition of a polysaccharide isolated from *E. cava*

Compounds	Contents (%)
Fucose	72.25
Galactose	12.28
Glucose	1.82
Mannose	4.74
Rhamnose	0.87
Xylose	8.04
Sulfate	6.17

1-3-2. SP did not show cytotoxicity on splenocytes

To establish that the splenocytes tested remained viable after incubation with SP for 24, 48, and 72 h, their presences were assessed by the MTT assay. As shown in Figure 1-2A, SP did not show the cytotoxicity on splenocytes at all concentrations (25, 50, and 150 μg/ml) and all incubation times (24, 48, and 72 h). In addition, the treatment of SP dose-dependently increased the cell viabilities at 24 and 48 h compared to control cells. These results indicate that SP has beneficial effects on the survival of immune cells.

1-3-3. SP enhanced proliferation of splenocytes

Next, to identify whether SP can lead to splenocytes' proliferation, a ^{3}H-thymidine incorporation assay was performed. In this assay, SP led to the proliferation of splenocytes at all concentrations (25, 50, and 150 μg/ml) and all incubation times (24, 48, and 72 h) tested, as compared to control cells (Figure 1-2B). Interestingly, SP's proliferative effects on splenocytes at 24 h were markedly higher than those of 48 and 72 h and it resulted in about six, seven, and thirteen-folds increased of splenocytes compared with control cells (***; $p <$ 0.001, ***; $p < 0.001$, ***; $p < 0.001$, respectively). These results showed that SP enhanced the proliferation of splenocytes.

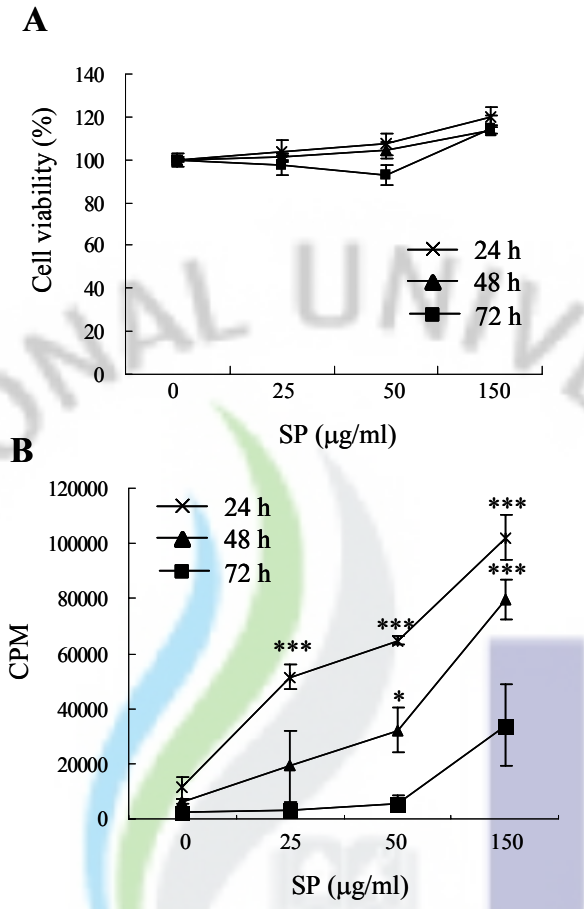

Figure 1-2. Effects of SP on viability (A) and proliferation (B) of splenocytes. (A) The effects of SP on cell viability were identified by MTT assay. (B) The proliferation of splenocytes was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.01$, ***, $p < 0.005$.

1-3-4. SP increased the populations of lymphocytes, monocytes, and granulocytes

To investigate the effects of SP on the three major cell types, namely, lymphocytes, monocytes and granulocytes, FACS analysis was used. As indicated in Figure 1-3A and B, SP markedly up-regulated the population and numbers of lymphocytes, monocytes and granulocytes, compared to those of control cells for 24 h (*; $p < 0.05$, *; $p < 0.05$, ***; $p < 0.001$, respectively). These results showed that SP stimulates the proliferation of lymphocytes, monocytes, and granulocytes

1-3-5. SP increased the proliferation of T and B lymphocytes, macrophages, and granulocytes

Next, the effects of SP on the specific cell population were measured by measuring forward-angle light scatter plotted against right-angle light scatter. As indicated in Figure 1-4, SP induced the increment in the populations and/or numbers of $CD3^+$ mature T lymphocytes, $CD45R/B220^+$ pan B lymphocytes, $CD11b^+$ macrophages and $Gr\text{-}1^+$ granulocytes. Especially, both the populations and numbers of $CD3^+$ mature T lymphocytes and $CD45R/B220^+$ pan B lymphocytes were markedly up-regulated by SP treatment in comparison with control cells (Figure 1-4A and B) (*; $p < 0.05$, *; $p < 0.05$, respectively). Also, SP significantly increased

the number of $CD11b^+$ macrophages and $Gr\text{-}1^+$ granulocytes, as compared to control cells (***; $p < 0.001$, *; $p < 0.05$, respectively). These results indicated that SP stimulates the proliferation of T and B lymphocytes, macrophages, and granulocytes.

1-3-6. SP enhanced the division of CFSE-labeled T and B lymphocytes, macrophages, and granulocytes

The effects of SP on the division of T and B lymphocytes, macrophages, and granulocytes were measured by identifying the progressive decrease in intensity of the CFSE dye which is transferred from parent cells to daughter cells in each succeeding immune cell generation. As shown in Figure 1-5, the divisions of CFSE-labeled $CD3^+$ mature T lymphocytes, $CD45R/B220^+$ pan B lymphocytes, $CD11b^+$ macrophages and $Gr\text{-}1^+$ granulocytes were increased by SP, compared to the only RPMI-treated CFSE-labeled cells (control cells). Especially, SP showed the significantly marked increment of CFSE-labeled daughter cells in $CD3^+$ mature T lymphocytes, $CD45R/B220^+$ pan B lymphocytes, $CD11b^+$ macrophages compared with control cells. These results suggested that SP enhanced the division of CFSE-labeled T and B lymphocytes, macrophages, and granulocytes

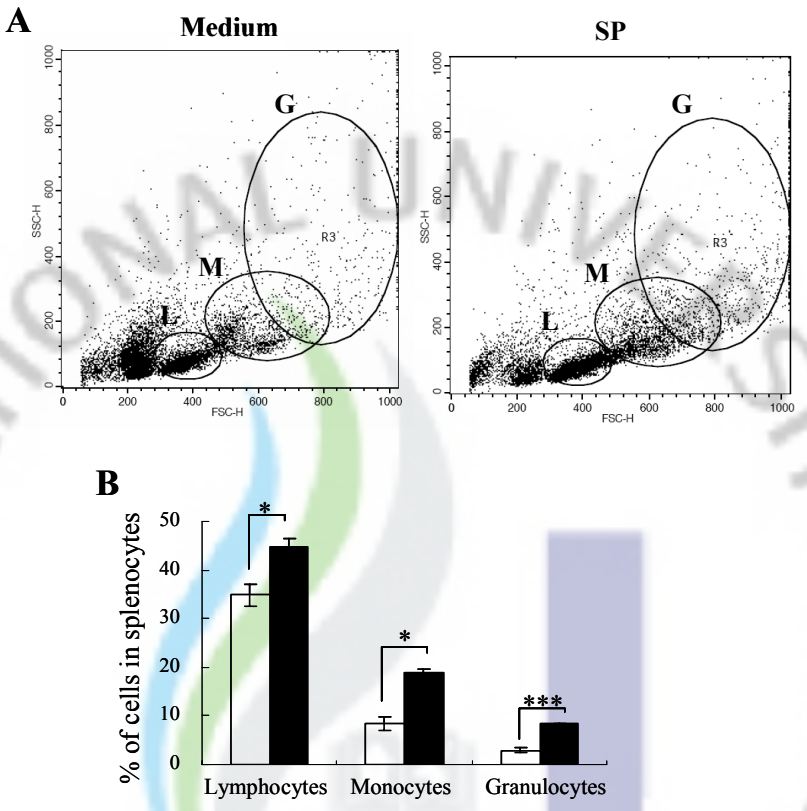

Figure 1-3. Gated lymphocytes, monocytes and granulocytes population analysis of proliferating splenocytes incubated with SP for 24 h. The cell populations including lymphocytes, monocytes and granulocytes were measured by FACS analysis. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$, **, $p < 0.005$.

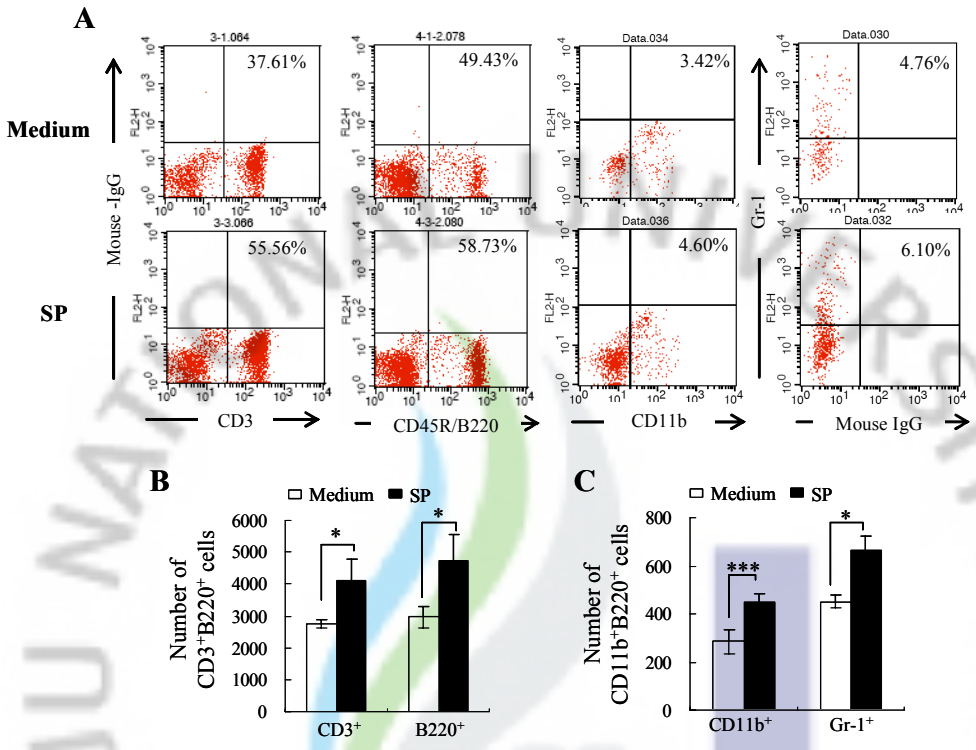

Figure 1-4. Effects of SP treatment on specific cell phenotypes in splenocytes. After 24 h of SP treatment, the specific cell phenotypes in splenocytes were measured by FACS analysis using anti-CD3, CD45R/B220, CD11b, and Gr-1 mAbs. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$, **, $p < 0.005$.

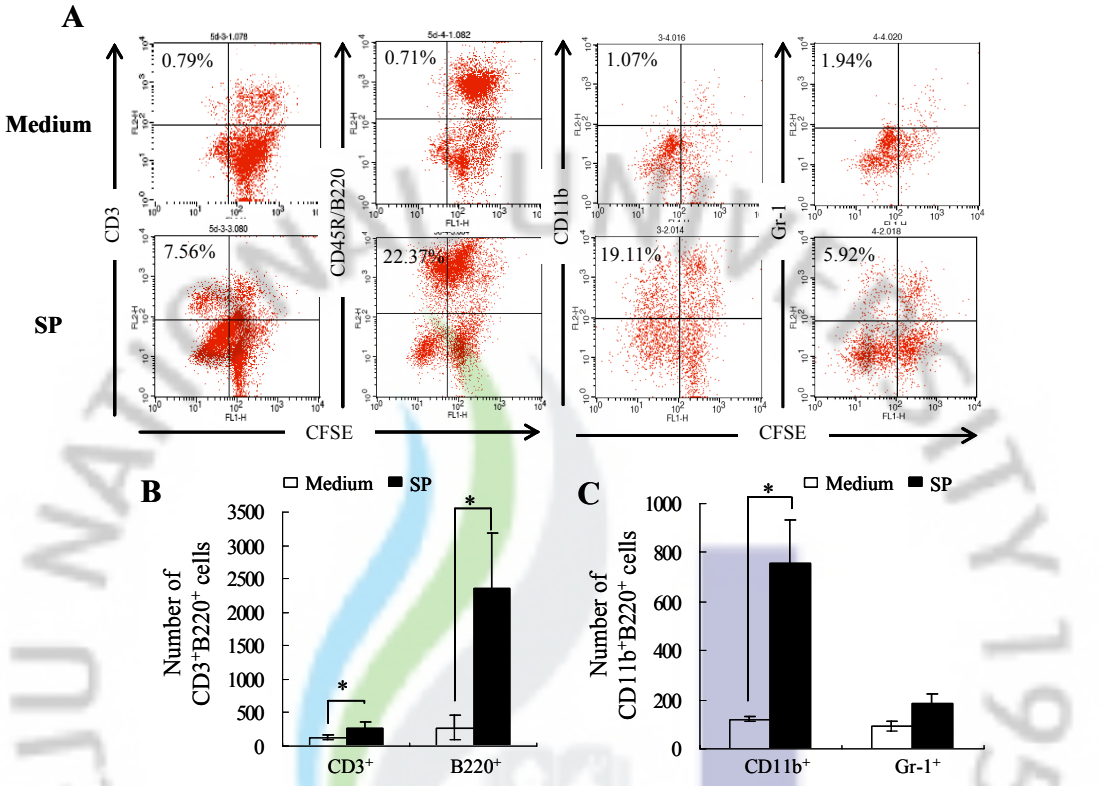

Figure 1-5. Effects of SP treatment on division of CFSE-labeled splenocytes. After 5 days of SP treatment, the division of CFSE-labeled cells was measured by FACS analysis using anti-CD3, CD45R/B220, CD11b, and Gr-1 mAbs. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$.

1-3-7. SP up-regulated the mRNA expression level and production of IL-2

Generally, IL-2 cytokine plays a key role in activation of Th1 and Th2 cells. So, the beneficial effects of SP on mRNA expression and production of IL-2 were identified by real time-PCR and the relative expression level of IL-2/GAPDH mRNA was calculated to normalize the level of IL-2 to GAPDH mRNA used as an internal control. Here, SP increased the relative mRNA expression of IL-2 in comparison with control cells (Figure 1-6A). Especially, the relative mRNA expression of IL-2 was gradually increased by SP treatment up to 12 h, and then declined for the next 24 h. However, its levels at all incubation times were higher than that of control cell.

Next, intracellular cytokine staining assay and ELISA was used to check the effect of SP on secretion and production of IL-2. As suggested in Figure 1-6B, SP did not show the significant difference on the population of $CD3^+$ mature T lymphocytes secreting IL-2, compared with control cells. However, the production of IL-2 was significantly up-regulated by the treatment of SP, comparing to control cells and it was higher about 2.5 folds than control cells (Figure 1-6C) (*; $p < 0.05$). These results suggested that IL-2 production and mRNA expression augmented by SP might induce the splenocyte's proliferation and division.

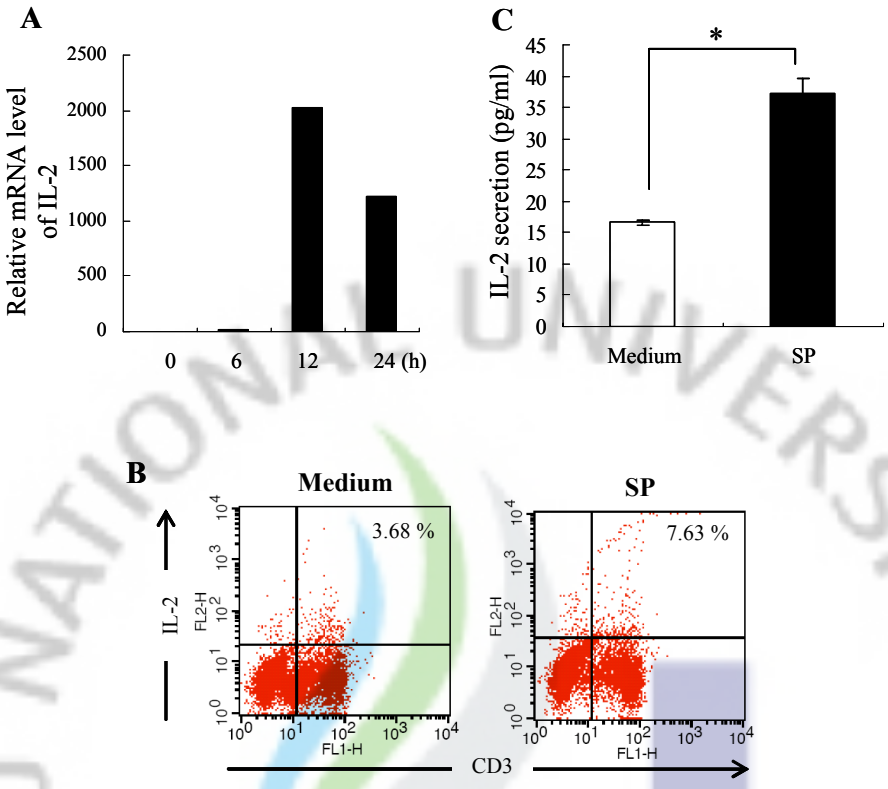

Figure 1-6. Effects of SP on mRNA expression (A), secretion (B), and production (C) of IL-2 in splenocytes. (A) The expression level of IL-2 in SP (150 μg/ml)-treated splenocytes was identified by RT- PCR. (B, C) The population of $CD3^{+}$-mature T lymphocytes secreting IL-2 and production of IL-2 in SP-treated splenocytes were measured by intracellular cytokine staining assay and ELISA. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$.

1-3-8. SP slightly increased the production of IgG1a and IgG2b in splenocytes

To identify the effects of SP on functional role of B lymphocytes, ELISA was used. The levels of IgG1a and IgG2b produced in SP-treated splenocytes were similar to those of control cells after 24 h of incubation (data not shown). But, at 3 days after treatment of SP, the production of IgG1a and IgG2b were slightly increased in splenocytes (*; $p < 0.01$, no significance, Figure 1-7). These results showed that SP induced functional roles of B lymphocytes, although their production was slightly increased in splenocytes.

1-3-9. Splenocyte's proliferation and IL-2 production induced by SP are blocked by the JNK inhibitor, SP600125

To investigate whether the proliferation of splenocytes and production of IL-2 induced by SP treatment is related with the activation of MAPK signal pathway including ERK, JNK and p38 molecules, ^{3}H-thymidine incorporation assay and ELISA was performed with the application of MAPK inhibitors which block the signal pathway of ERK, JNK and p38 molecules. As indicated in Figure 1-8, SP induced the marked increment in the proliferation of splenocytes, comparing to that of control cells (***; $p < 0.001$). However, the application of SP600125, a JNK inhibitor significantly reduced the proliferation of splenocytes increased by SP (Figure 1-8A) (***; $p < 0.001$), although other inhibitors did not affect to it.

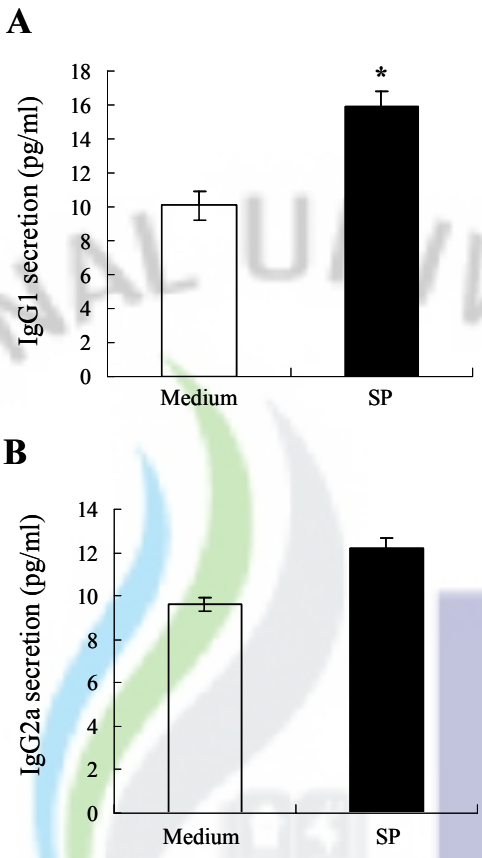

Figure 1-7. Effects of SP treatment on IgG1 (A) and IgG2a (B) in splenocytes. Splenocytes were treated or not with SP (150 μg/ml) for 72 h and the supernatants were used to measure the concentrations of IgG1 and IgG2a using commercially available ELISA kits. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$.

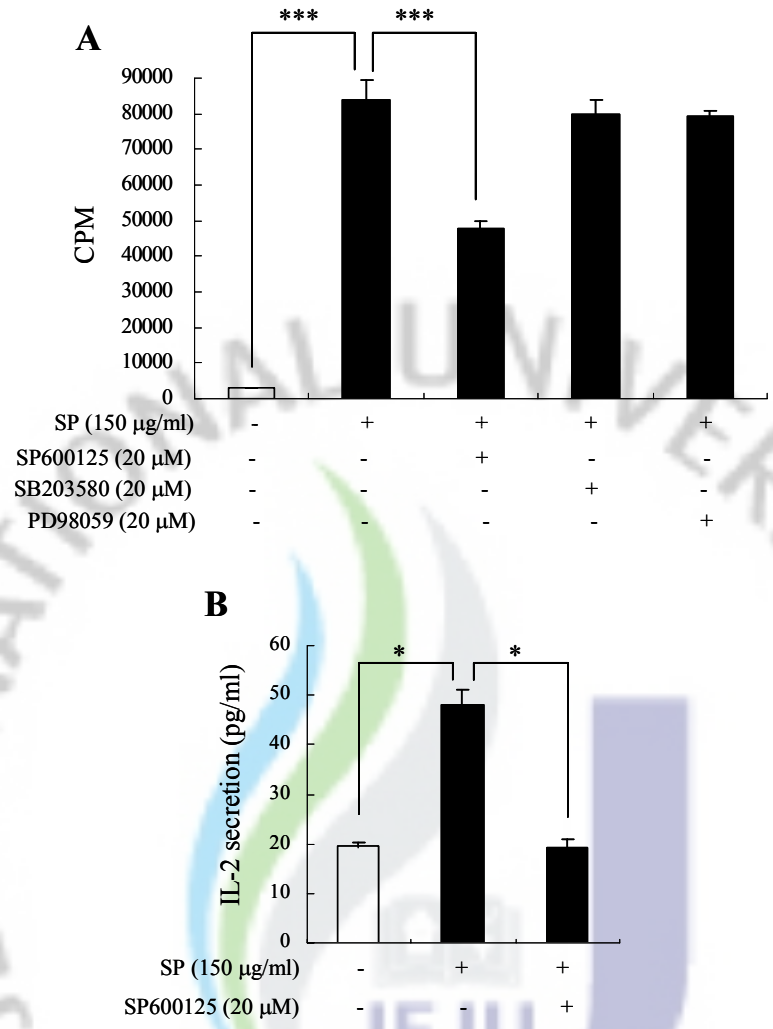

Figure 1-8. Blockage by MAPK inhibitors including JNK, p38, and ERK on the proliferation (A) and total IL-2 production (B) of splenocytes induced by SP. (A) After incubation with SP for 24 h, the supernatants were used for ELISA analysis. (B) The splenocyte's proliferation was measured by the incorporation of ^{3}H-thymidine. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$, ***, $p < 0.005$.

In addition, the IL-2 production stimulated by SP in splenocytes was markedly blocked by the application of JNK inhibitor (Figure 1-8B) (*; $p < 0.05$, *; $p < 0.05$). These results indicate that the activation of JNK signal pathway might be required for the capacities of SP on splenocyte's proliferation and cytokine production.

1-3-10. Activation of JNK/c-fos signal pathway induced by SP was considerably blocked by the JNK inhibitor, SP600125

The effects of SP on the activation of JNK signal pathway was examined by Western blot and EMSA. In illustrated in Figure 1-9A and B, SP considerably induced the phosphorylation of JNK as well as the activation of nuclear c-fos at 0.5 h after stimulation, followed by reduction for the next 24 h compared to levels in control cells, whereas SP did not affect to activation of nuclear c-jun (data not shown). β-actin was used as internal control. Moreover, the AP-1 DNA binding was markedly activated by SP treatment at same time point, comparing to control cells, and then declined at 1 h (Figure 1-9C). Interestingly, the activation of JNK/c-fos signal pathway induced by SP treatment was inhibited by the application of SP600125, a JNK inhibitor as decreased the phosphorylation of JNK, the activations of nuclear c-fos and AP-1-DNA binding (Figure 1-10). These results indicate that the activation of JNK/c-fos signal pathway is required for the splenocyte's proliferation and

cytokine production stimulated by SP.

1-3-11. Splenocyte's proliferation and IL-2 production induced by SP was inhibited by blockage of NFκB signal pathway

Normally, NFκB signal pathway known as downstream molecule of JNK leads to survival, proliferation, and differentiation of cells and cytokine production (Pimentel-Muiños et al., 1994; Armitage RJ et al., 1995; Gerondakis et al., 1998; Varga et al., 1999; Yamamoto et al., 2001; Prasad et al., 2002; Granucci et al., 2003; Mora et al., 2003). So, to investigate whether the capacities of SP on splenocyte's proliferation and IL-2 production is mediated by NFκB signal pathway, ^{3}H-thymidine incorporation assay and ELISA was performed with the application of NFκB inhibitors such as TPCK, a serine protease inhibitor or PDTC, a NFκB inhibitor. As shown in Figure 1-11A and B, the proliferation of splenocytes markedly increased by SP treatment was significantly inhibited by the application of TPCK or PDTC (***; $p < 0.001$, *; $p < 0.05$). Also, the application of TPCK or PDTC considerably decreased the IL-2 production stimulated by SP (Figure 1-11C and D) (*; $p < 0.05$, *; $p < 0.05$). These results suggest that splenocyte's proliferation and IL-2 production induced by SP were inhibited by blockage of NFκB signal pathway.

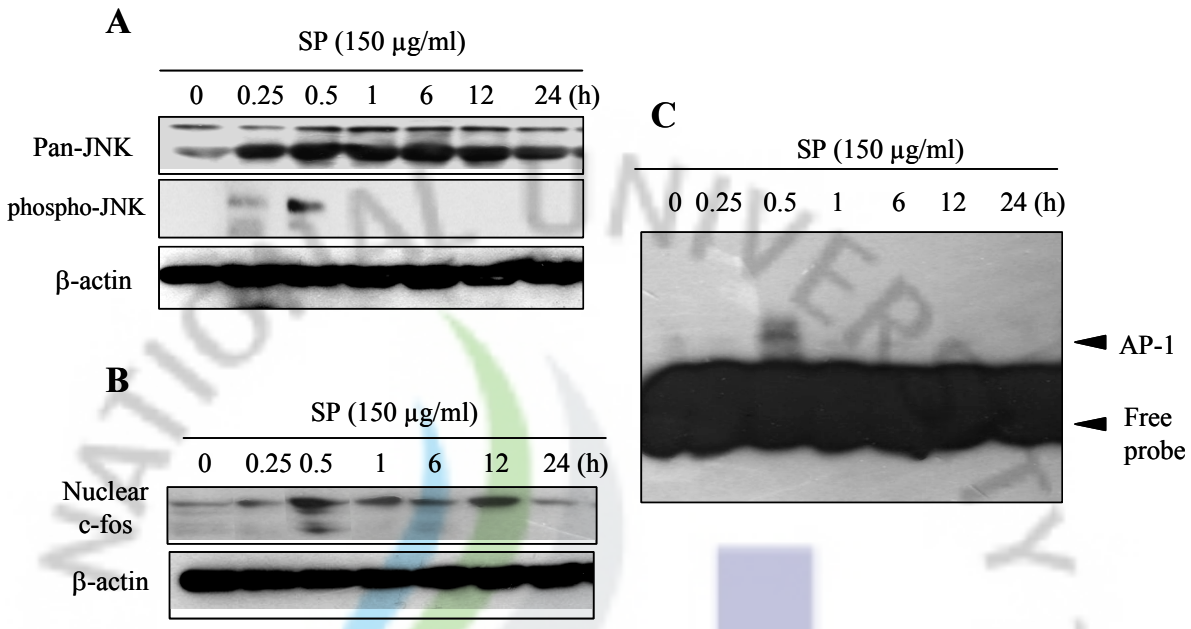

Figure 1-9. Effects of SP on expressions of cytoplasmic pan-JNK and phospho-JNK (A), nuclear c-fos (B), and nuclear AP-1 DNA binding activity (C). The cytoplasmic and nuclear proteins were extracted from splenocytes incubated with SP (150 μg/ml) for 0.25, 0.5, 1, 6, 12, and 24 h. The cytoplasmic (50 μg) (A) and nuclear (40 μg) (B) proteins were immunoblotted with pan-JNK, phosphor-JNK, c-fos, and β-actin antibodies. (C) Nuclear proteins (10 μg) were assayed for Ap-1 activation as described in Materials and Methods.

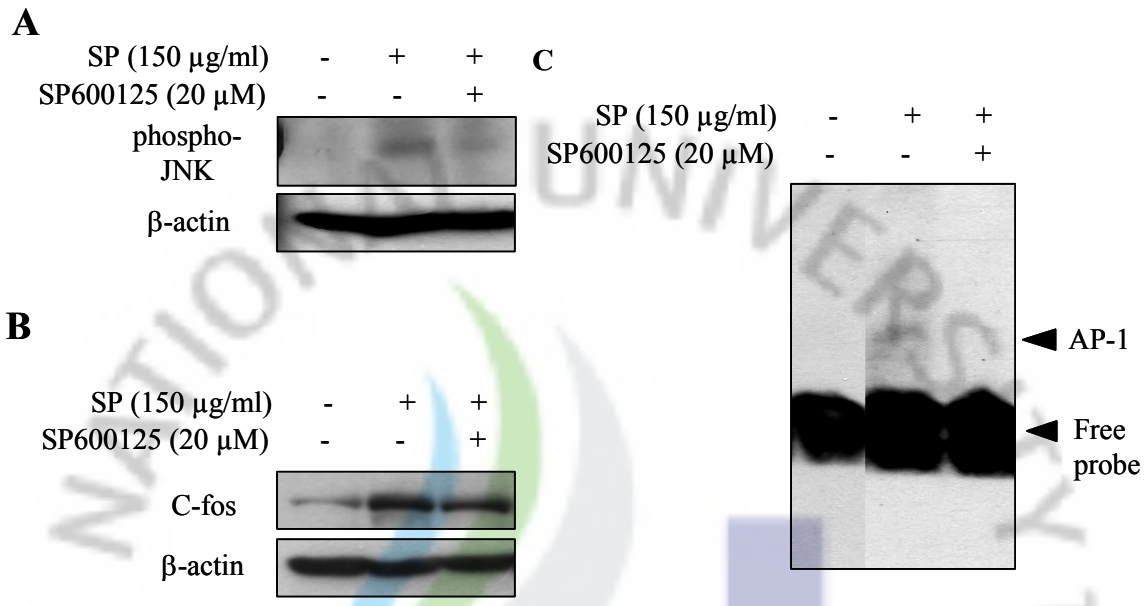

Figure 1-10. Blockage by SP600125, a JNK inhibitor on expressions of cytoplasmic phospho-JNK (A), nuclear c-fos (B), and nuclear AP-1 DNA binding activity (C). The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with SP (150 μg/ml) and SP600125 (20 μM) for 0.5 h. The cytoplasmic (50 μg) (A) and nuclear (40 μg) (B) proteins were immunoblotted with phospho-JNK, c-fos, and β-actin antibodies. (C) Nuclear proteins (10 μg) were assayed for Ap-1 activation as described in Materials and Methods.

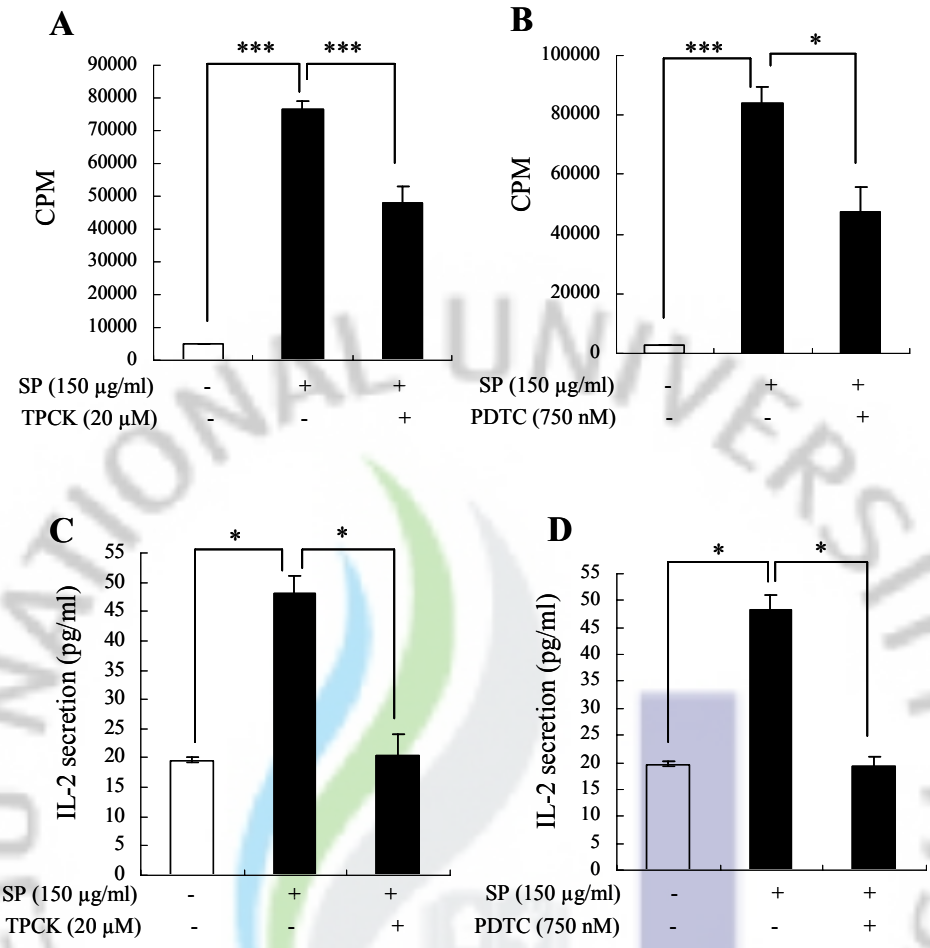

Figure 1-11. Blockage by TPCK and PDTC known as inhibitors of NFκB on the proliferation (A and B) and total IL-2 production (C and D) of splenocytes induced by SP. Splenocytes was incubated with SP, TPCK, and/or PDTC. After 24 h, the proliferation and IL-2 production of splenocytes were measured by ^{3}H-thymidine incorporation assay and ELISA. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. *, $p < 0.05$, ***, $p < 0.005$.

1-3-12. SP induced the phosphorylation of IκBα, and translocation of NFκB p65 into nuclei as well as NFκB p65 DNA binding

As Figure 1-12A illustrates, the stimulation of SP gradually increased the phosphorylation of cytoplasmic IκBα with time dependent manner up to 24 h of incubation. However, it slightly decreased the expression of IκBα at 0.25 h after SP treatment compared to control cells and then recovered from 0.5 h (Figure 1-12A).

Next, as indicated in Figure 1-12B, SP gradually led to the translocation of NFκB p65 into nucleus in proliferated splenocytes in comparison with control cells from 0.25 to 1 h, followed by reduction for the next 6 h compared to levels in control cells. But, the translocation of NFκB p65 into nucleus was again markedly increased by SP at 12 h and then complicatedly declined at 24 h. In addition, NFκB p65 DNA binding was gradually increased by the stimulation of SP from 0.25 to 1 h, followed by reduction at 6 h and then again increased from 12 to 24 h (Figure 1-12C). These results indicated that SP indeed led to the proliferation of splenocytes via inducing the phosphorylation of IκBα, and translocation of NFκB p65 into nuclei as well as NFκB p65 DNA binding, all of which constitute typical cascade of events along the classical NFκB pathway.

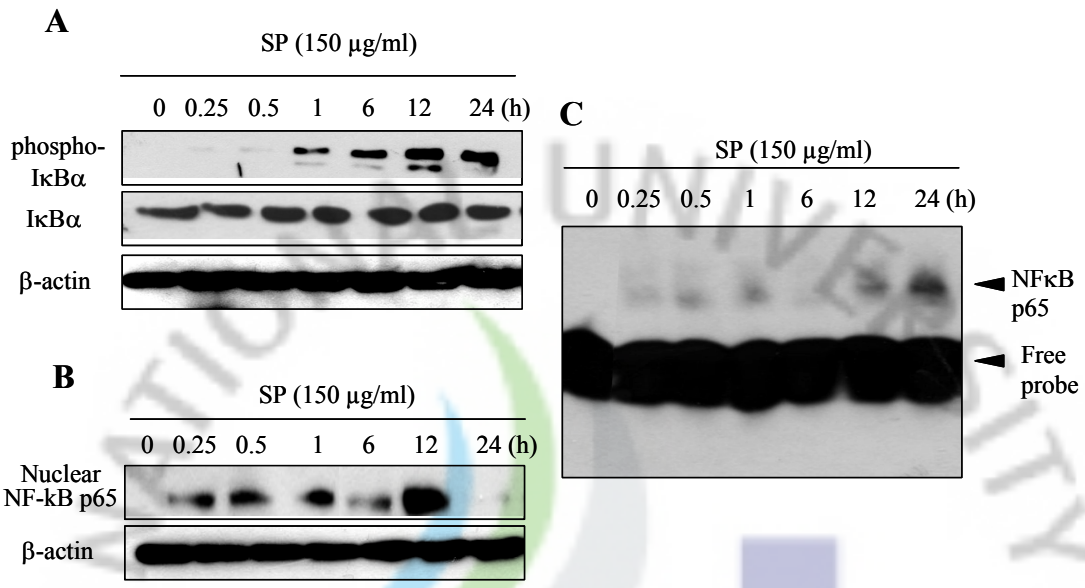

Figure 1-12. Effects of SP on expressions of cytoplasmic IκBα and phospho-IκBα (A), nuclear NFκB p65 (B), and nuclear NFκB p65 DNA binding activity (C). The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with SP (150 μg/ml) for 0.25, 0.5, 1, 6, 12, and 24 h. The cytoplasmic (50 μg) (A) and nuclear (40 μg) (B) proteins were immunoblotted with IκBα, phospho-IκBα and β-actin antibodies. (C) Nuclear proteins (10 μg) were assayed for NFκB p65 activation as described in Materials and Methods.

1-3-13. Activation of the classical NFκB pathway induced by SP was markedly blocked by the NFκB inhibitors

To reveal whether the NFκB inhibitors such as TPCK and PDTC can directly inhibit the activation of the classical NFκB pathway induced by SP, Western blot and EMSA were performed. In Western blot analysis, we showed that the application of TPCK and PDTC reduced the phosphorylation of IκBα as well as activation of nuclear NFκB p65 induced by SP (Figure 1-13A and B). Especially, the application of PDTC completely inhibited against the phosphorylation of IκBα and activation of nuclear NFκB p65 induced by SP (Figure 1-13B). Also, the treatment of PDTC (750 nM) completely inhibited NFκB p65-DNA binding activated by SP in comparison with only SP-treated cells (Figure 1-13C), whereas TPCK slightly inhibited it. These results clearly substantiate the contention that the classical NFκB p65 pathway is mainly involved in the activation, proliferation, and differentiation of lymphocytes.

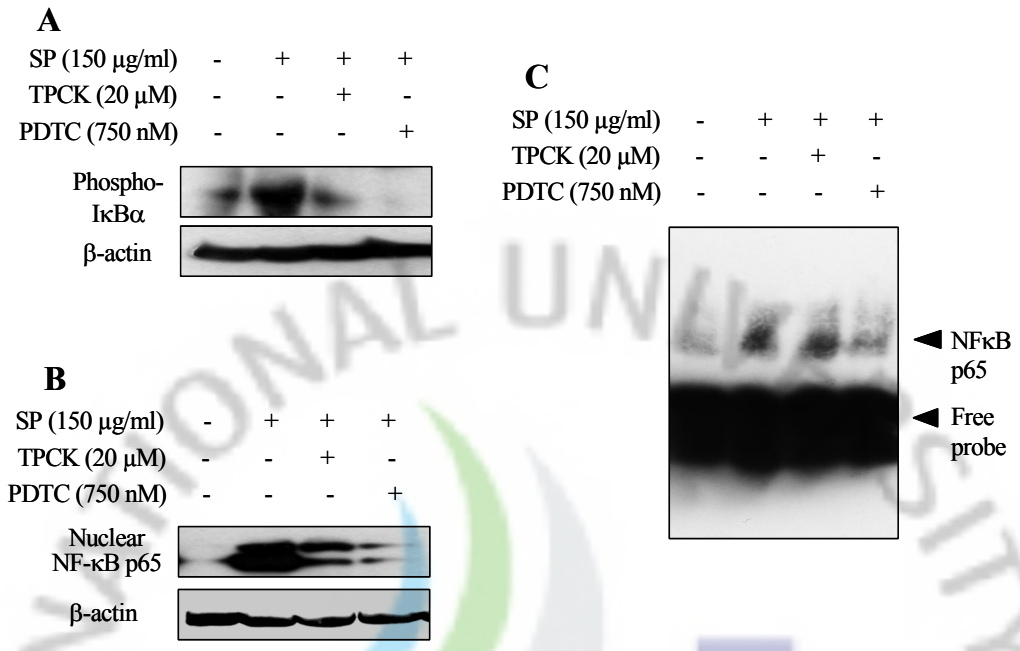

Figure 1-13. Blockage by TPCK and PDTC known as inhibitors of NFκB on expressions of cytoplasmic IκBα and phospho-IκBα (A), nuclear NFκB p65 (B), and nuclear NFκB p65 DNA binding activity (C). The cytoplasmic and nuclear proteins were extracted from the splenocytes incubated with SP, TPCK and/or PDTC for 12 h. The cytoplasmic (50 μg) (A) and nuclear (40 μg) (B) proteins were immunoblotted with IκBα, phospho-IκBα and β-actin antibodies. (C) Nuclear proteins (10 μg) were assayed for NFκB p65 activation as described in Materials and Methods.

1-4. Discussion

This present study demonstrates that a marine algal polysaccharide (SP) isolated from *E. cava* known as a fucoidan containing plentiful fucose and sulfated group contents elicits the activation of splenocytes through activating the JNK/NFκB signaling

During the last decade, an upsurge of information has surfaced concerning the immunological role of plant-derived materials used in transitional medicine. Especially, many previous studies have reported the immunological capacities such as immunolodulatory, anti-inflammatory and immunostimulant properties of polysaccharides of plants, i.e., *Actibidia eriantha*, *Lobophora variegate* and *Capsosiphon fulvescens* (Xu et al., 2009; Sun et al., 2009; Medeiros et al., 2008; Hwang et al., 2008). Thus, the previous study had also shown that *E. cava* containing plentiful polysaccharide induced the activation, proliferation and differentiation of murine splenocytes as stimulating IL-2 production through the activation of classical NFκB pathway (Ahn et al., 2008 and 2010). Interestingly, this present study showed that SP, a major compound of *E. cava* significantly stimulated the proliferation and differentiation of splenocytes containing lymphocytes, monocytes and granulocytes without cytotoxicity as well as the synthesis, production, and activation of IL-2. Recently, previous studies indicated that various polysaccharides isolated from plants and seaweeds led to the production of cytokine including IL-1, IL-6, IFN-γ and TNF-α and the

activation of MAPKs and/or NFκB signaling pathway via toll like receptor (TLR)4 as lipopolysaccharide (LPS) for few years (Shao et al., 2004; Desai et al., 2007). Also, MAPK, ERK1/2, and ERK5 are important in the control of IL-2 (Varga et al., 1999; Dumont et al., 1998), transcription factors such as NFκB (Garaude et al., 2005, 2006) and MEK/ERK signaling pathway is required for the survival of T and B lymphocytes during mitogenic stimulation (O'Reilly et al., 2009; Liu et al., 2004). In addition, the protein complex that binds to the Ap-1 transcriptional activation of the IL-2 gene promoter region may comprise c-Jun-Fos heterodimer, and formation of the AP-1 complex is critical for regulation of IL-2 gene expression (Schafer et al., 2003; Angel et al., 1991; Jain et al., 1992; Jain et al., 1992; Meugge et al., 1989; Serfling et al., 1989). The present study also showed the applications of direct antagonists of JNK and NFκB activation markedly inhibited the SP-stimulated splenocyte's proliferation and IL-2 production. Moreover, SP indeed led to the activation of JNK, c-fos and NFκB p65 as well as AP-1- and NFκB p65-DNA binding in nucleus, although did not affect to that of c-Jun. Also, this study showed that the application of SP600125, TPCK, and PDTC directly led to the marked inhibition on the activation of JNK/c-fos and NFκB signal pathway induced by SP treatment. These results suggest that the classical NFκB signal pathway via the activation of JNK/c-fos signal pathway is required for splenocyte's proliferation and IL-2 production stimulated by SP. Also, these results indicate

that SP might respond to TLR4 for the activation, proliferation, and differentiation of splenocytes via activating JNK/c-fos and NFκB signal pathways.

In conclusion, this study suggests that the activation of splenocytes induced by SP is dependent on the classical NFκB pathway via JNK/c-fos activation as the augmentation of IL-2 expression and production. Also, future studies are expected to investigate the affects of TLR4 on the activation of JNK/c-fos and NFκB signal pathways induced by SP in splenocytes.

PART 2

Protective effects of sulfated polysaccharide isolated from *Ecklonia cava* against damages caused by gamma ray irradiation in murine splenocytes

Abstract

Background: Exposure of gamma ray irradiation inhibits survival and proliferation of cells as causing DNA damage via the production of ROS which induces oxidative stress. Previous studies have reported that *Ecklonia cava* (Alariaceae, Phaeophyta) containing plentiful polysaccharide has the beneficial capacities for antioxidant, immunomodulation and immune activation. Therefore, this present study investigated whether sulfated polysaccharide (SP) of *E. cava* containing the high content of fucose and sulfate group has possible impact on the reduction of radiation-induced tissue damages and, if effective, its underlying mechanism *in vitro*.

Methods: At 24 or 72 h after exposure of 2 Gy irradiation, the effects of SP on the survival and proliferation of splenocytes known as radio sensitive cells were identified by MTT and ^{3}H-thymidine incorporation assay. Also, to examine the effect of SP on reactive oxygen species (ROS) production in 2 Gy-irradiated splenocytes, DCF-DA (2'-7'-dichlorofluorescein

diacetate) assay was performed. And then, to investigate the cytoprotective effects of SP on DNA damages such as the increment of tail DNA percentage (%) and olive tail DNA movement (um) and the formation of apoptotic DNA in sub-G1 phase, Comet assay and Propidium iodide (PI) staining assay were used in 2 Gy-irradiated splenocytes. In further study, at 12 h after exposure of 2 Gy irradiation, the effects of SP on the expression of apoptosis-related molecules (Bcl-2, Bcl-xL, and Bax) were performed in splenocytes.

Results: In this study, SP significantly stimulated the survival and proliferation of splenocytes known as a radio sensitive cell after exposure of 2 Gy irradiation. In addition, SP treatment significantly reduced the production of ROS caused by gamma ray irradiation. Also, DNA damages such as tail DNA percentage (%) and olive tail DNA movement (um) and the formation of apoptotic sub-G_1 hypodiploid cells caused by gamma ray irradiation were markedly decreased by treatment of SP in splenocytes with increment of concentrations. Moreover, SP inhibited apoptosis via reducing the expression level and of Bax and increasing those of Bcl-2 and Bcl-xL after exposure of gamma ray irradiation.

Conclusion: In conclusion, these results demonstrate that SP leads to radio protective effects against DNA damages by apoptosis through modulating apoptosis-related molecules in gamma ray irradiated splenocytes. Additionally, it might be used for a potential candidate for radiotherapy of cancer patients.

2-1. Introduction

Marine algae that can be divided into three basic types: brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta) seaweed have been reported to contain diverse classes of biologically active compounds which are useful in the pharmaceutical industry. In addition, they contain large amount of polysaccharides, such as alginate, fucoidan, laminaran, agarose, and carrageenan and polyphenols such as phloroglucinol, eckol, and dieckol. Among them, especially, many researchers have studied about the biological roles of various polyphenolic compounds such as phloroglucinol, eckol, and dieckol and reported their beneficial effects as potential materials for radioprotection, antioxidant, and anticancer activity for few years (Kang et al., 2005a, b, and 2006a, b; Park et al., 2008a, b, and 2010; Ahn et al., 2007; Kang et al., 2004; Kong et al., 2009). However, in recent years, there has been increasing interest in investigating the biological capacities of marine algal polysaccharides known as another major active compound in brown seaweeds. Indeed, previous studies have demonstrated that polysaccharides isolated from brown seaweeds such as *Laminaria japonica*, *Undaria pinnitafida*, and *Sargassum thunbergii* have antitumor, anticoagulant, antioxidant effects *in vitro* and *in vivo* (Gueniche F et al., 2006; Hu et al., 2010; Wang et al., 2010; Zhuang et al., 1995). Also, recent studies have suggested that acidic polysaccharide from *Panax Ginseng* and fucoidan has radioprotective and

immunomodulatory effect (Kim et al., 2009a, b; Lim et al., 2004; Park et al., 2009; Lee et al., 2005; Lee et al., 2008; Byon et al., 2008; Kim et al., 2007). At these points, evaluating the beneficial functions of new polysaccharides isolated from seaweed is needed to development of functional food or drugs industry.

Recently, many researchers have reported that *Ecklonia cava (E. cava)*, a kind of brown seaweed has biological efficacy such as reactive oxygen (ROS) scavenging (Kang et al., 2004a; Ahn et al., 2007), bactericidal effect (Nagayama et al., 2002), anti-plasmin inhibiting (Fukuyama et al., 1989), antimutagenesis (Lee et al., 1998), and cell damage inhibitors (Kang et al., 2005a, b; Ahn et al., 2007). Until now, however, researchers have rarely probed the biological roles of polysaccharide in *E. cava* and, if effective, its underlying mechanism. Additionally, the recent studies have demonstrated that *E. cava* containing plentiful polysaccharide led to immunomodulation and immune activation of murine splenocytes (Ahn et al., 2008a and 2010), there is worth investigating the polysaccharide's beneficial effects on immune response. Moreover, previous studies have indicated that the potential compounds showing biological effects such as antioxidant, immunomodulation, and immune activation have induced radio-protective effects against exposure of gamma ray irradiation in vitro or in vivo (Kang et al., 2005a,b, 2006a,b; Park et al., 2008a,b; Ahn et al., 2007). Therefore, the polysaccharide isolated from *E. cava* might be

a potential material for development of natural radio protector.

Here, this study indicates that sulfated polysaccharide from *E. cava* (SP) has radio protective effects as enhancing the survival and proliferation of murine splenocytes as well as inhibiting apoptosis by modulating apoptotic protein expressions.

2-2. Materials and Methods

2-2-1. Preparation of enzymatic extract from *E. cava*

E. cava collected from the coast of Jeju Island, South Korea was washed with fresh water, freeze-dried and pulverized into powder with a grinder. Ten gram of the dried *E. cava* was homogenized with 1 L of distilled water (pH 6.0) and mixed with 1 mg of AMG (Novo Nordisk, Bagsvaerd, Denmark). The reaction was conducted at 40°C for 12 h and the digest was boiled for 10 min at 100°C to inactivate the enzyme. The product was clarified by 20 min of centrifugation at 3000 × *g* to remove any unhydrolyzed residue. After filtration of the enzymatic extract, the supernatant was adjusted to pH 7.0, freeze-dried and then stored for use in experiments.

2-2-2. Isolation of polysaccharide from *E. cava* (SP) and its composition

To isolate a polysaccharide from the enzymatic extract, ethanol (2 L) was added into the enzymatic extract (1 L) and the reaction was kept in 4℃ for 24 h. After centrifugation, a polysaccharide was isolated from the precipitation and freeze-dried for 3 days. Then, the compositions of SP were analyzed by measuring the contents of sulfate group and monosaccharides such as fucose, xylose, galactose, mannose, and glucose known as the standard markers of various polysaccharides according to a modified version of methods

indicated in the previous study (Saito et al., 1968; Ahn et al., 2008). The isolated polysaccharide showed the large amount of fucose (about 72.25%) and sulfate group (about 6.17%) and the other composition's contents was little. From this results, the polysaccharide can be regarded as a sulfated polysaccharide (SP) due to its highly fucose and sulfate group contents.

2-2-3. Isolation of splenocytes from spleen

Spleens were aseptically removed from C57/BL6 mice, ages 6 to 9 weeks (SLC, Inc., Shizuoka, Japan), and red blood cells were lysed by immersion in ACK lysis buffer including 0.84% ammonium chloride at room temperature for 10 min in darkness. After washing with Dulbecco's phosphate-buffered saline (DPBS) (Gibco BRL, Life Technologies, New York, USA), the purified splenocytes were suspended in RPMI (Roswell Park Memorial Institute) medium including 10% fetal bovine serum (FBS) and 1% antibiotic (100 U/ml penicillin and 100 mg/ml streptomycin) (Sigma, St. Louis, MO, USA) to use directly for experiments.

2-2-4. Irradiation with ^{60}Co γ-rays

Splenocytes were exposed to γ-irradiation from a ^{60}CO Theratron® (Best Theratronics Ltd, Ottawa, Ontaria, Canada) teletherapy unit at Applied radiological Science research Institute, Jeju National University, Korea, at a dose rate of 1.5 Gy/min.

2-2-5. MTT assay

The effect of SP on the survival of splenocytes after exposure of gamma ray irradiation (2 Gy) was examined by an MTT assay known as a colorimetric assay that is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mossmann, 1983). The non-irradiated or irradiated splenocytes (1×10^5 cells/wells) were incubated with or without SP at various concentrations (from 25 to 150 μg/ml) for 24 h. Control cells were treated with only RPMI medium. Then, MTT stock solution (10 μl; 5 mg/ml) was applied to each of the wells for 4 h. The absorbance of formazan crystals dissolved in 100 μl of solublization buffer (pH 4.7) including 50% dimethylformamide (DMSO) and 10% sodium dodecyl sulfate (SDS) was measured at 540 nm using enzyme-linked immunosorbent assay (ELISA) plate reader. The optical density of the formazan generated in control cells was considered to represent

100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

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

A ^{3}H-thymidine incorporation assay known as a 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, USA) was performed to determine whether SP stimulates the proliferation of splenocytes inhibited by gamma ray irradiation. For this assay, the 4×10^{5} cells were cultured with SP at final concentrations of 25, 50, or 150 μg/ml in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). Concanavalin A (2 μg/ml) was used for positive control cells. After incubation for 72 h at 37°C, 95% humidity and 5% CO_2, 1 μCi of ^{3}H-thymidine (specific activity 42 C_i/mmol, Amersham, Arlington Heights IL, U.S.A.) was added to the cells, and the plates were incubated for an 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 (Wallac MicroBeta® TriLux, PerkinElmer, Waltham, MA, U.S.A.).

2-2-7. DCF-DA (2'-7'-dichlorofluorescein diacetate) assay

To detect the production levels of intracellular reactive oxygen species (ROS), DCF-DA assay was performed. After irradiation of 2 Gy, splenocytes were directly seeded onto a 96 well culture plate at 1×10^5 cells/well and incubated with SP at concentrations of 25, 50, and 150 μg/ml for 30 mins. After incubation, 25 μM of DCF-DA solution was added to each well for 10 min at 37℃. The intensity of 2, 7-dichlorofluorescein was measured at 585 nm and 620 nm using a Perkin Elmer LS-5B spectrofluorometer (Becton Dickinson, Mountain View, CA, U.S.A.). The intracellular ROS scavenging activity (%) was calculated as 100 × [(optical density of irradiated group) - (optical density of irradiated group with SP treatment)] / (optical density of irradiated group).

2-2-8. Comet assay

The alkaline comet assay was conducted to determine whether SP inhibits DNA damages of cells induced by gamma ray irradiation according to Singh et al. (Singh et al., 1995)'s method with a little modification. 2 Gy-irradiated splenocytes were incubated with SP at concentrations of 25, 50, and 150 μg/ml for 24 h. After incubation, the cells (5×10^4 cells) were washed using DPBS and then, suspended in 100 μl of 0.7% low melting point agarose (LMPA). After keeping them for 15 min at 4℃ for solidification of the agarose, the

cells were lyzed in lysis solution (2.5 M NaCl, 100 μM EDTA, 10 mM Tris, 1% sodium laurylsarcosine and 1% Triton X- 100) for 1 h at 4℃. The slides were applied for electrophoresis with the electric current of 25 V/300 mA for 20 min. Then, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 10 min two times and dehydrated with 70% ethanol. DNA tail percentages (tail intensity, TI; 50 cells from each of two replicate slides) and olive tail movements (μm) of each cell were measured by Komet 5.5 software (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany).

2-2-9. Propidium iodide (PI) staining assay

PI staining assay was used to determine whether SP reduces the proportion of apoptotic sub-G_1 hypodiploid cells in irradiated splenocytes. After irradiation of 2 Gy, splenocytes (1×10^5 cells) were incubated with SP at all concentrations of 25, 50, or 150 μg/ml for 6, 12, or 24 h. The cells were harvested at the indicated times and fixed in 1 mL of 70% ethanol for 30 min at 4°C. Then, the cells were incubated in darkness in 500 ul of DPBS containing 50 μg PI (Sigma) and 50 μg RNase A (Sigma) at 37°C. After 30 min, samples were analyzed by assessing the proportion of sub G_1 cells using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, U.S.A.)

2-2-10. Western blot analysis

The effects of SP on the expression of apoptosis-related molecules such as p53, Bax, and Bcl-2 were assessed by Western blot analysis. Splenocytes irradiated with 2 Gy were cultured with SP at all concentrations of 25, 50, and 150 μg/ml. After 24 h, cytoplasmic protein was prepared from the cells by using NE-PER^{R} Nuclear and Cytoplasmic Extraction Reagents according to manufacturer's instructions. Cytoplasmic (50 μg/well) preparation was loaded into SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and electrophoresed under denaturing conditions. Subsequently, proteins were electro-transferred onto nitrocellulose transfer membrane. After blocking with 5% nonfat milk for 2 h, blots were incubated with primary antibodies such as p53 (1:1000 dilution, Cell Signaling Technology Inc., U.S.A), Bax (1:1000 dilution, Cell Signaling Technology Inc.), Bcl-2 (1:1000 dilution, Cell Signaling Technology Inc.) or β-actin (1:3000 dilution, Sigma) antibodies for 60 min followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin (Ig) G (Cell Signaling Technology Inc.) for 60 min. Visualization was achieved by using X-ray film and chemiluminescence reagents.

2-2-11. Statistical analysis

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

2-3. Results

2-3-1. SP increased the survival of splenocytes reduced by gamma ray irradiation

As shown in Figure 2-1A, exposure of gamma ray irradiation decreased the survival of splenocytes, compared to non-irradiated cells at 24 h. However, the treatment of SP significantly increased the survival of splenocytes inhibited by gamma ray irradiation at all concentrations (25, 50, and 150 μg/ml). Especially, the survival of cell increased by 150 μg/ml of SP was similar with that of non-irradiated cells. These results indicate that SP has beneficial effects on the survival of splenocytes exposed by gamma ray irradiation.

2-3-2. SP enhanced the proliferation of splenocytes without cytotoxicity

Next, to determine whether SP stimulates splenocyte's proliferation against gamma ray irradiation, a ^{3}H-thymidine incorporation assay was performed. In this assay, the proliferation of splenocytes was markedly reduced by exposure of gamma ray irradiation as compared to non-irradiated cells (***; $p < 0.001$) (Figure 2-1B). Interestingly, the treatment of SP (25, 50, and 150 μg/ml) resulted in about two, four, and five-folds increased of splenocytes in comparison with 2 Gy-irradiated cells (***; $p < 0.001$, ***; $p < 0.001$, ***; $p < 0.001$). These results suggested that SP stimulated the splenocyte's proliferation against

gamma ray irradiation.

2-3-3. SP inhibited the production of intracellular ROS caused by gamma ray irradiation

After irradiation of 2 Gy, DCF-DA assay was used to investigate whether SP reduces the production levels of intracellular ROS in splenocytes. As illustrated in Figure 2-2, irradiation of 2 Gy slightly increased the production levels of intracellular ROS in splenocytes, compared with that of non-irradiated cells. However, when SP was treated into the 2 Gy-irradiated cells, the increased intracellular ROS production was markedly decreased by 150 μg/ml of SP (***; $p < 0.001$), although 25 and 50 μg/ml of SP did not show significant difference. These results indicate that SP might increase splenocyte's survival and proliferation by inhibiting the production of intracellular ROS caused by gamma ray irradiation

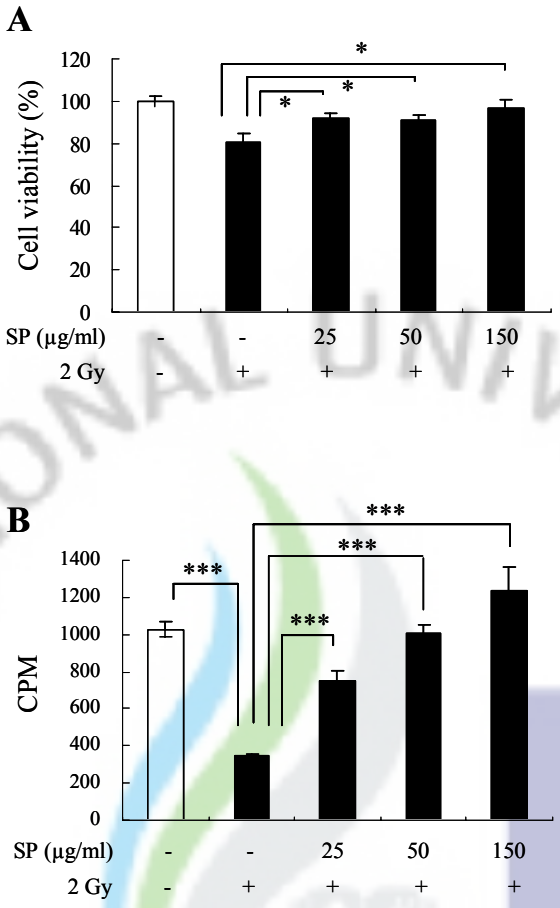

Figure 2-1. Effects of SP on the survival (A) and proliferation (B) of 2 Gy-irradiated splenocytes. (A) One hundred thousand cells were exposed to 2 Gy irradiation and treated with SP (25, 50, and 150 μg/ml). After 24 h, the survival of cells was identified by MTT assay. (B) Four hundred thousand viable cells were exposed to 2 Gy irradiation and treated with SP (25, 50, and 150 μg/ml). After 72 h, the 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.E. *, $p < 0.01$, ***, $p < 0.005$.

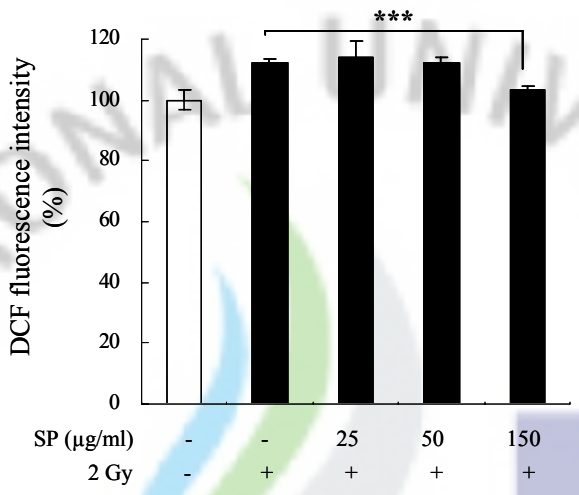

Figure 2-2. Effects of SP on the intracellular ROS produced in gamma ray-irradiated splenocytes. The production levels of intracellular ROS were measured by DCF-DA assay. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. ***, $p < 0.005$.

2-3-4. SP protected splenocytes against DNA damages caused by gamma ray irradiation

Next, to further identify whether SP inhibits DNA damages of cells induced by gamma ray irradiation, the alkaline comet assay was performed. Figures 2-3A, B, F, and G showed that gamma ray irradiation markedly caused DNA damages as increasing tail DNA percentage (%) and olive tail DNA movement (μm) in splenocytes, comparing to that of non-irradiated cells. But, SP treatment markedly inhibited DNA damages caused by gamma ray-irradiation, compared to only 2 Gy-irradiated cells. In addition, SP treatment dose-dependently decreased tail DNA percentage (%) from 54.22 to 34.99% and olive tail DNA movement (um) from 54.22 to 34.99 μm in 2 Gy-irradiated cells (***; $p < 0.001$, ***; $p < 0.001$, and ***; $p < 0.001$, respectively) (Figures 2-3F and G), comparing with only 2 Gy-irradiated cells. These results indicate that SP protected splenocytes by inhibiting DNA damages caused by gamma ray irradiation.

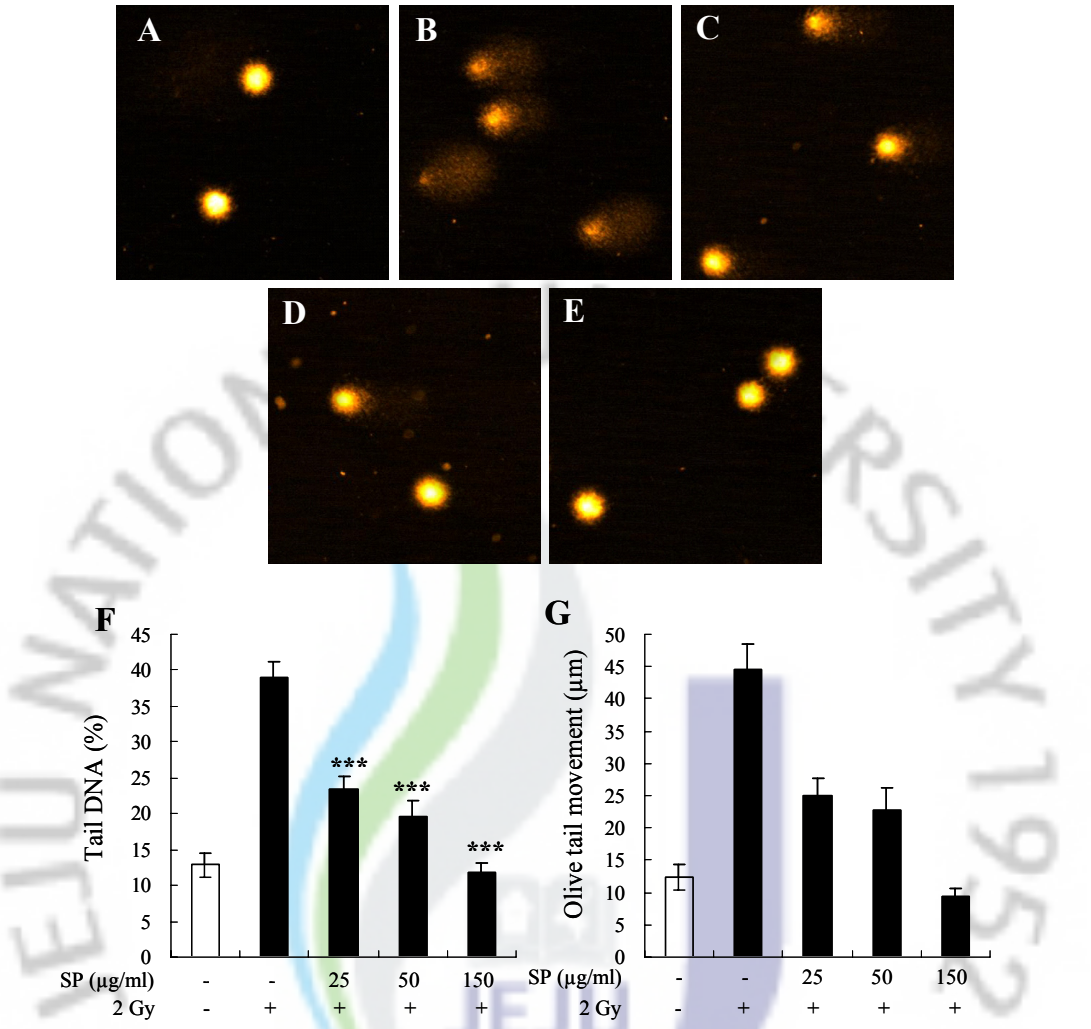

Figure 2-3. Image of DNA damages (A-E) and the effects of SP on the tail DNA percentages (F), and olive tail DNA movement (G) in caused by gamma ray irradiation in splenocytes. (A) Non-irradiated cells, (B) 2 Gy-irradiated splenocytes, (C) SP 25 μg/ml treated and irradiated splenocytes, (D) SP 50 μg/ml treated and irradiated splenocytes, (E) SP 150 μg/ml treated and irradiated splenocytes. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. **, $p < 0.01$, ***, $p < 0.005$.

2-3-5. SP reduced the population of apoptotic DNA caused by gamma ray irradiation in splenocytes

To identify whether SP decreases the proportion of apoptotic sub-G_1 hypodiploid cells in 2 Gy-irradiated splenocytes, PI staining assay was used. As indicated in Figures 2-4A, B and F, the formation of apoptotic DNA in Sub-G_1 peak was dramatically increased at 24 h after gamma ray irradiation up to 75.57%, compared to non-irradiated cells (36.59%). Whereas, SP treatment showed an interestingly lower percentage of cells in apoptotic peak at all concentration (25, 50, and 150 μg/ml), when compared with only gamma ray-irradiated cells. Moreover, it was decreased following the increment of SP's concentrations (60.27, 47.23, and 31.34%, respectively in SP-treated and irradiated cells *vs* 75.57% in 2 Gy-irradiated cells) (Figure 2-4, ***; $p < 0.001$, ***; $p < 0.001$, **; $p < 0.01$, respectively).

In addition, at 6, 12, and 24 h after exposure of gamma ray-irradiation, the populations of apoptotic DNA were time-dependently increased up to 11.98, 45.91, and 71.50%, respectively (***; $p < 0.001$, ***; $p < 0.001$, ***; $p < 0.001$, respectively) (Figure 2-5). However, when 150 μg/ml of SP was treated into the 2 Gy-irradiated cells, the increment of apoptotic DNA caused by gamma ray irradiation were significantly decreased from 11.98, 45.91, and 71.50% to 5.13, 16.44, and 29.95% at all incubation times. These data suggest that SP led to a cytoprotective effect against gamma ray irradiation-induced apoptosis.

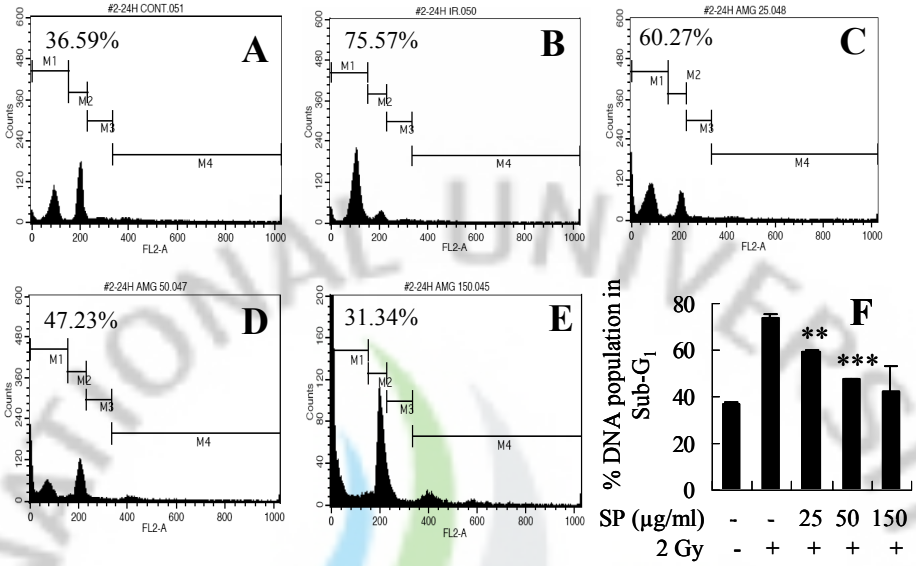

Figure 2-4. Effects of SP on the formation of apoptotic DNA caused in 2 Gy-irradiated splenocytes. The formation of apoptotic DNA in sub-G_1 phase of splenocytes was identified by PI staining assay. (A) Histogram of non-irradiated cells, (B) Histogram of 2 Gy-irradiated splenocytes, (C) Histogram of SP 25 μg/ml-treated and irradiated splenocytes, (D) Histogram of SP 50 μg/ml-treated and irradiated splenocytes, (E) Histogram of SP 150 μg/ml-treated and irradiated splenocytes, (F) Column on the formation of apoptotic DNA in SP-treated and irradiated splenocytes. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. **, $p < 0.01$, ***, $p < 0.005$.

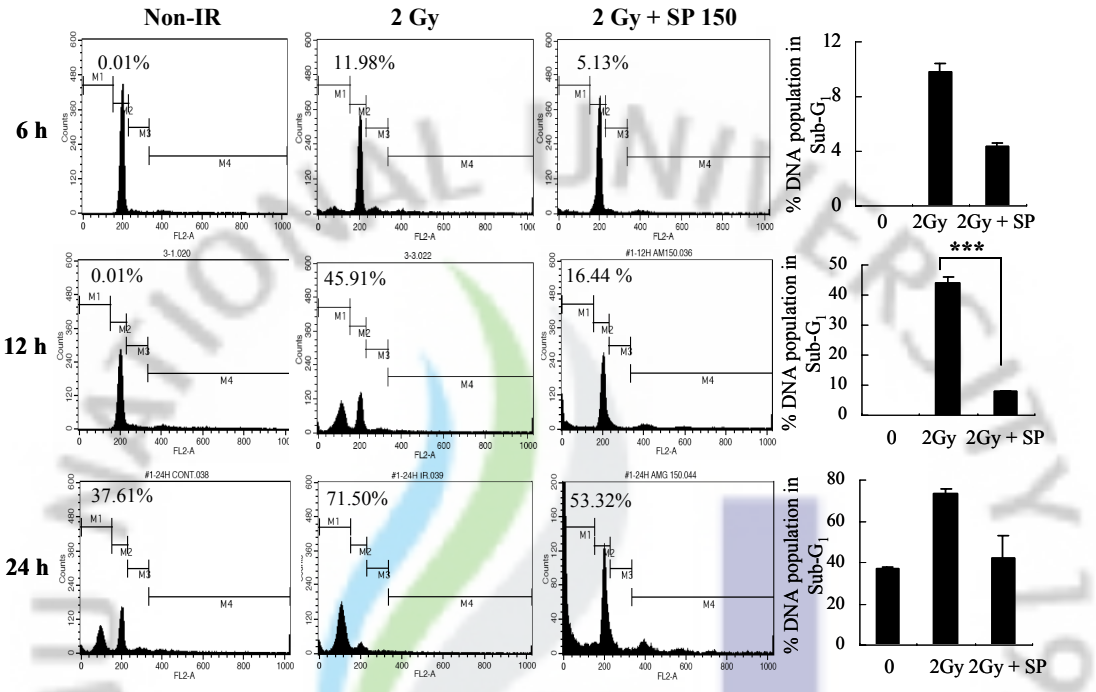

Figure 2-5. Effects of SP on the formation of apoptotic DNA caused in 2 Gy-irradiated splenocytes. Splenocytes were exposed to 2 Gy irradiation and treated with SP (150 μg/ml). After 6, 12, and 24 h, the formation of apoptotic DNA in sub-G_1 phase of splenocytes was identified by PI staining assay. Experiments were performed in triplicates, and data are expressed as average percent change from untreated controls ± S.E. ***, $p < 0.005$.

2-3-6. SP modulated the expression levels of apoptosis-related proteins in 2 Gy-irradiated cells

To assess whether SP can inhibit apoptosis by regulating mitochondrial signal pathway including Bax, Bcl-2 and Bcl-xL in gamma ray-irradiated splenocytes, Western blot assay was performed. As shown in Figure 2-6, exposure of gamma ray (2 Gy) irradiation considerably up regulated the expression level of Bax known as a pro-apoptotic molecule, whereas markedly down regulated Bcl-2 and Bcl-xL known as anti-apoptotic molecules in splenocytes, comparing with only 2 Gy-irradiated cells. However, SP treatment dose-dependently decreased the expression levels of Bax, a pro-apoptotic molecule at all concentrations from 25 to 150 μg/ml compared to only 2 Gy-irradiated cells. In addition, the expression levels of Bcl-2 and Bcl-xL were markedly up-regulated at all concentrations comparing with 2 Gy-irradiated cells. Especially, 150 μg/ml of SP markedly decreased Bax expression, whereas increased the Bcl-2 and Bcl-xL expressions in comparison with only2 Gy-irradiated cells. These data indicates that SP inhibited apoptosis caused by gamma ray irradiation as modulating apoptosis-related molecules.

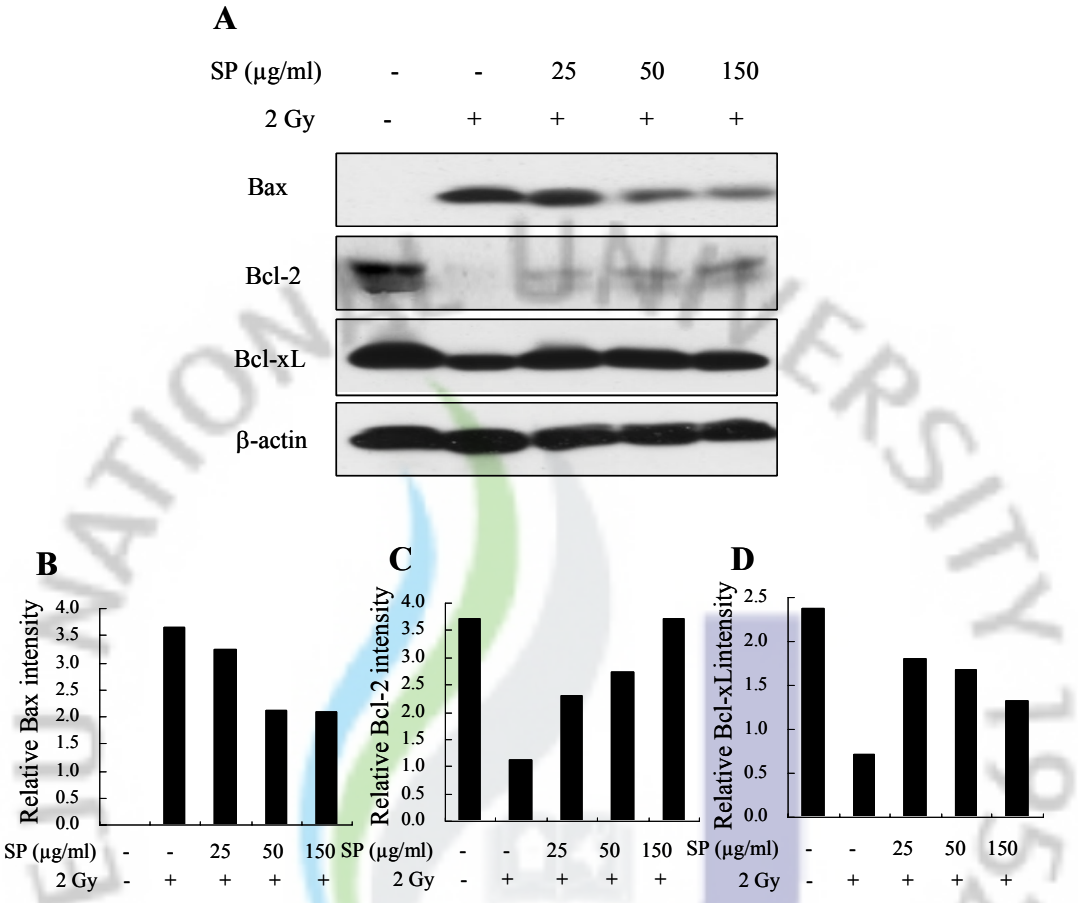

Figure 2-6. Effects of SP on the expression levels of apoptosis related molecules in 2 Gy-irradiated splenocytes (A) and its densitometric analysis (B-D). Splenocytes were exposed to 2 Gy irradiation and treated with SP (25, 50, and 150 μg/ml). After 24 h, the expression levels of apoptosis related molecules such as Bax, Bcl-2, and Bcl-xL were determined by Western blot. Experiments were performed in triplicate.

2-4. Discussion

Exposure of gamma ray irradiation inhibits survival and proliferation of cells by causing DNA damage followed by the production of ROS which induces oxidative stress. In addition, when mice or human was exposed to gamma ray irradiation, it causes the destruction of the lymphoid and hemopoietic systems by inducing apoptosis of proliferating stem cells and splenocytes known as peripheral immune cells. So, many researchers have studied whether natural products protect peripheral immune cells or stem cells against DNA damages and apoptosis caused by gamma ray irradiation (Bing et al., 2010; Byon et al., 2008; Checker et al., 2008; Subramanian et al., 2005). Here, sulfated polysaccharide (SP) isolated from *E. cava* showed radio protective effects against damages by gamma ray irradiation in peripheral immune cells. Also, this study indicated that SP significantly increased the survival and proliferation of murine splenocytes after exposure of gamma ray irradiation. Previous studies have indicated that ROS can lead to functional damage in lipid, proteins and DNA, which can eventually result in cell death (Kang et al., 2005a,b, 2006a,b). In this study, SP inhibited DNA damage by decreasing ROS production in gamma ray irradiated splenocytes. Recently, many researchers have reported that *E. cava* protects cells against irradiation- or H_2O_2-induced cell damage via cellular antioxidant activation by modulating expression of apoptosis related with proteins for few years (Kang et al., 2005a,b

and 2006a,b; Prak et al., 2008b and 2010). In addition, the previous study has demonstrated that fucoidan extract derived from *Cladosiphon novae-caledoniae* effectively reduced both intracellular and released H_2O_2 of HT1080 cells (Ye et al., 2005). Our previous study has also shown that *E. cava* has antioxidant effects by scavenging the production of ROS and inhibiting cell damage induced by H_2O_2 (Ahn et al., 2007; Heo et al., 2005). These results indicated that SP's scavenging capacity on ROS leads to cytoprotective effects as decreasing DNA damage such as DNA strand breakage and apoptotic DNA caused by gamma ray irradiation. Also, it suggests that SP's inhibitory effects on ROS production and DNA damage might be related with the modulation of apoptotic molecules such as Bax, a pro-apoptotic molecule and Bcl-2 and Bcl-xL, anti-apoptotic molecules. Several studies have established that oxidative stress-induced apoptosis is associated with expressions of Bax, Bcl-2 and Bcl-xL in murine splenocytes and chinese hamster lung fibroblast (Kang et al., 2005a,b and 2006a,b; Prak et al., 2008b, Bing et al., 2010). As a result of the present study, SP inhibited apoptosis by decreasing the expression of Bax, whereas increasing those of Bcl-2 and Bcl-xL in gamma ray-irradiated splenocytes.

In conclusion, these results suggest that SP has radio protective effects by enhancing the survival and proliferation of cells as decreasing ROS production and DNA damage via Bcl-2/Bax signal pathway in gamma ray irradiated splenocytes. However, additional studies on

signaling pathway related to the radio-protective capacities of SP are needed.

PART 3

Protective effects of sulfated polysaccharide isolated from *Ecklonia cava* against immune suppression caused by gamma ray irradiation in mice

Abstract

Background: Gamma ray irradiation causes the destruction of lymphoid and hemopoietic systems by increasing apoptosis of proliferating stem cells and peripheral immune cells. Previous studies have reported that *Ecklonia cava* (Alariaceae, Phaeophyta) (*E. cava*) containing plentiful polysaccharide has the beneficial effects for antioxidation, immunomodulation and immune activation. Here, this study investigated whether sulfated polysaccharide (SP) of *E. cava* has possible impact on the reduction of radiation-induced tissue damages without toxicities and, if effective, its underlying mechanism *in vivo*.

Methods: First, SP's toxicities were assessed in single doses (2000 and 5000 mg/kg, by oral route, for 14 days) and sub acute toxicity tests (1, 10, and 100 mg/kg, by oral route, for 21 days). At the end of two tests, hematological changes were determined by using the automatic hematocyte analyzer, which included red blood cell (RBC), hemoglobin

concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell (WBC). Also, the weights of all organs such as liver, Kidney, Spleen, and Lung were measured. Next, to assess SP's radio protective effects, SP (10 mg/kg b.w.) was injected intraperitoneally twice into mice first at 18 h and then again at 2 h before irradiation. At 1 day or 5 days after gamma ray (2~9 Gy) irradiation, alkaline comet assay, ^{3}H-thymidine incorporation assay, and flow cytometry assay were performed to see whether SP can stimulate the proliferation and differentiation of peripheral immune cells as inhibiting their DNA damages. Then, to investigate SP's hematopoietic capacities at 9 days after gamma ray irradiation, the bone marrow cell counting, colony forming unit (CFUs) assays, and hematoxylin and eosin (H&E) staining were used. In addition, to examine the biological mechanism, Western blot and immunohistochemistry (IHC) were performed to check the expression and immunoreactivity of apoptosis-related molecules (Bcl-2, Bax, and p53) in intestine. Finally, their survival rates for 30 days were investigated to define the radioprotective effects of SP in lethally irradiated mice.

Results: In single dose and sub acute toxicity tests, SP recorded no deaths or hazardous signs did not affect to the body and organs weight in all groups, comparing to control groups. In addition, SP did not show significant difference in hematological parameters among the

control and treated groups. Next, SP significantly stimulated the proliferations of peripheral blood lymphocytes and splenocytes via inhibiting DNA damages in irradiated mice. Also, SP treatment significantly accelerated the hemopoietic recovery of bone marrow cells and peripheral splenocytes as well as the regeneration of intestinal crypt cells, compared to untreated control. Furthermore, SP inhibited apoptosis in intestine by reducing the expression levels and immunoreactivities of p53 and Bax and increasing those of Bcl-2 after irradiation. Finally, this study observed prolonged survival of SP treated irradiated mice compared to irradiated but untreated controls.

Conclusion: Our results demonstrate that SP did not show toxicities in mice and leads to radio protective effects by enhancing immunostimulatory and hematopoietic capacities and inhibiting apoptosis in gamma ray-irradiated mice.

3-1. Introduction

The exposure of gamma ray irradiation causes the destruction of the lymphoid and hemopoietic systems via increasing apoptosis of not only proliferating stem cells including bone marrow and intestinal crypt cells, but also splenocytes including lymphocytes (Andreson et al. 1976). However, it has been used as a worthy tool in a wide variety of fields; i.e. agriculture, medicine research, military operations, and therapy of cancer patients (Moon et al., 2008). So, for few years, many researchers have reported that the various products has the radioprotective effects as improving lymphoid or hemopoietic systems by decreasing apoptosis (Park et al., 2008a,b, 2009 and 2010; Byon et al., 2008; Bing et al., 2010). Nevertheless, Federal Drug Administration (FDA) has approved only few products that can alleviate the injurious effects of ionizing radiation safely and effectively (Park et al., 2010) due to their toxic side effects. Therefore, the screening of natural products that have no toxic for radio protective drugs has been required. The previous studies have documented the radioprotective effects of various natural products, i.e. *Elaeocarpus sylvestris*, *Ecklonia cava* and *Panax ginseng* against radiation (Park et al., 2008a,b,c and 2009, 2010; Kang et al., Lee et al., 2005; Kim et al., 2007). Also, recent studies have been reported that acidic polysaccharide and fucoidan have radioprotective and immunomodulatory effects *in vitro* and *in vivo* (Kim et al., 2007; Kim et al., 2009a; Lim et al., 2004; Lee et al., 2008; Byon et

al., 2008; Kim et al., 2008). Interestingly, the radio protectors including polysaccharides have been reported that they also have the beneficial capacities on antioxidant, immunomodulation, and immune activation. Therefore, searching the potential active compounds showing antioxidant, immunomodulation, and immune activation might contribute to development of new natural radio protectors. From these points, the polysaccharide isolated from *E. cava* might be a potential material for development of natural radio protector, because in the previous study, *E. cava* containing plentiful polysaccharide induced immunomodulation and immune activation in immune cells (Ahn et al. 2008, 2010). Until now, however, researchers have rarely probed the polysaccharide's possible impact on the reduction of radiation-induced tissue damages and, if effective, its underlying mechanism *in vivo*.

In the present study, sulfated polysaccharide from *Ecklonia cava* (SP) has radio protective capacity as enhancing the proliferation and differentiation of immune cells as well as improving the regeneration of stem cells through modulating expression of apoptosis-related molecules in mice.

3-2. Materials and methods

3-2-1. Mice

C57BL/6 mice were purchased from Orientbio, Inc. (Sungnam, Korea). The mice were housed in conventional animal facilities with a NIH-07-approved diet and water ad libitum at a constant temperature (23 ± 1°C) according to the guidelines for the Care and Use of Laboratory Animals of the Institutional Ethical Committee of Jeju National University. Mice were used for the experiments when 6-8 weeks of age and 18-25 g of weight. Mice were randomly separated into three groups: a sham irradiated group, an irradiated control group, and SP plus irradiation group.

3-2-2. Preparation of enzymatic extract from *E. cava*

E. cava collected from the coast of Jeju Island, South Korea was washed with fresh water, freeze-dried and pulverized into powder with a grinder. Ten gram of the dried E. cava was homogenized with 1 L of distilled water (pH 6.0) and mixed with 1 mg of AMG (Novo Nordisk, Bagsvaerd, Denmark). The reaction was conducted at 40°C for 12 h and the digest was boiled for 10 min at 100°C to inactivate the enzyme. The product was clarified by 20 min of centrifugation at 3000 × g to remove any unhydrolyzed residue. After filtration of

the enzymatic extract, the supernatant was adjusted to pH 7.0, freeze-dried then stored for use in experiments.

3-2-3. Isolation of polysaccharides from *E. cava* (SP)

To isolate a polysaccharide from the enzymatic extract, ethanol (2 L) was added into the enzymatic extract (1 L) and kept in 4℃ for 24 h. After centrifugation, a polysaccharide (SP) was isolated from precipitation and it was freeze-dried for 3 days. Then, the compositions of SP were analyzed by measuring the contents of sulfate group and monosaccharides such as fucose, xylose, galactose, mannose, and glucose known as the standard markers of various polysaccharides according to a modified version of methods indicated in the previous study (Saito et al., 1968; Ahn et al., 2008).

3-2-4. Single dose toxicity test

For single dose toxicity of SP in mice, female and male C57BL/6 mice (5 mice/group) were divided into one control group and three concentrations of SP treated groups 1, 10, and 100 mg /kg body weight following the guidelines of Korea Food & Drug Administration (KFDA). The control group received saline. And, each treated group 6 times received SP for 6 h at first day and then daily by the oral route from 2 to 14 days. The mice were daily

observed for up to 14 days following treatment for behavioral changes and signs of toxicity and/or death and the latency of death. At the end of the experiment (15 days), the heparinized bloods were obtained from the mice of all groups and used for a hematological determination by using the automatic hematocyte analyzer, which included red blood cell (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell (WBC).

3-2-5. Sub-acute toxicity test

For sub-acute toxicity of SP in mice, female and male C57BL/6 mice (5 mice/group) were divided into one control group and two concentrations of SP treated groups 2000 and 5000 mg /kg body weight following the guidelines of Korea Food & Drug Administration (KFDA). The control group received saline. And, each treated received daily by the oral route from 1 to 21 days. After sacrifice, their behavioral changes, signs of toxicity and/or death and the latency of death were daily observed and at 22 days, the weights of all organs such as liver, Kidney, Spleen, and Lung were measured. Additionally, the hematological determination and the biochemical parameters were assessed by identical methods indicated in above.

3-2-6. Treatment of SP

The SP dissolved in saline was injected intraperitoneally (i.p.) twice into mice, receiving the 10 mg/kg b.w. dose first at 18 h and then again at 2 h before irradiation. Additionally, as controls, sham irradiated and irradiated mice were injected i.p. with saline at the same volume as test mice.

3-2-7. Irradiation with ^{60}Co γ-rays

Each mouse was placed individually in a close-fitting Perspex box (3×3×11 cm) and given a single dose of WBI at dose rate of 1.5 Gy / min at a source-surface distance of 150 cm from a ^{60}Co irradiator (Theratron-780 Teletherapy unit, Applied Radiological Science Institute, Jeju National University).

3-2-8. Preparation of primary splenocytes and peripheral blood lymphocytes

Mice were killed by cervical dislocation, and single-cell suspensions were prepared by pressing the spleens through a cell strainer as described (Ahn et al., 2008). The purified splenocytes were suspended in RPMI-1640 medium (Gibco-BRL) that was supplemented 10% fetal bovine serum (FBS) (Gibco-BRL) and 100 U/ml antibiotics (Gibco-BRL). And

then, the purified cells were measured by Trypan blue dye exclusion (Sigma Aldrich, St. Louis, MI, USA), and the cells (viability > 90%) were used directly for the additional experiments.

Next, peripheral blood lymphocytes were isolated from the whole blood in mice of each group (3 mice/group) by using Ficoll-Hypaque (Sigma-Aldrich). The cells were used for comet assay.

3-2-9. Comet assay

To determine the cytoprotective effects of SP on oxidative DNA damage induced in peripheral blood lymphocytes and splenocytes, an alkaline comet assay was used. At 1 day after exposure to 2 Gy of ionizing radiation, peripheral blood lymphocytes and splenocytes isolated from mice of each group (3 mice/group) were used for this assay. The cells were lyzed in lysis buffer (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, and 1% Triton X-100, pH 10) for 1 hr at 4°C. After electrophoresis, the DNAs were observed under a fluorescence microscope and analyzed by using the Komet 5.5 program (Kinetic Imaging, Liverpool, UK). The percentage of fluorescence in the tail DNAs of 100 cells per slide was recorded. DNA damage-inhibition activity (%) was calculated as: [(the percentage of fluorescence in the tail DNA of irradiated group - the percentage of fluorescence in the tail DNA of SP-treated and

irradiated group)/the percentage of fluorescence in the tail DNA of irradiated group] × 100.

3-2-10. ^{3}H-thymidine incorporation assay

To assess whether SP stimulates the proliferation of splenocytes damaged by gamma ray irradiation, a ^{3}H-thymidine incorporation assay was performed. At 5 days after 2 Gy irradiation, the splenocytes (4×10^{5} cells) obtained from three mice used in each group were cultured with RPMI-1640 medium supplemented 10% FBS and 100 U/ml antibiotics at 37 °C in humidified air containing 5% CO_2. For a positive control, concanavalin A (Con A) (2 μg/well) (Sigma) was added to appropriate wells. After incubation for 3 days, each well was pulsed for a final 18 h with 1 μCi of ^{3}H-thymidine (specific activity 42 Ci/mmol; Amersham, Arlington Heights, IL, USA). The cells were harvested onto glass fiber filters (Amersham), and the amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer (MicroBeta TriLux, Perkin Elmer, Wallac, Germany). The results were expressed as CPM.

3-2-11. Flow cytometry assay

To further define the specific cell populations stimulated by SP, the specific population of the cells was measured by FACS analysis. Mice (3 mice/group) were sacrificed at 5 days

after 2 Gy WBI, and their spleens were removed. The splenocytes (1×10^6) were harvested and twenty thousand viable cells per mouse (as determined by light scatter profiles) were analyzed by using a BD FACS Calibur™ flow cytometer (BD Biosciences). The cytogram indicated three main clusters related to the major cell types of the splenocytes; lymphocytes, monocytes, and polymorphonuclear granulocytes. Splenocytes (1×10^6) were harvested and, to inhibit nonspecific staining, combined with an anti-mouse immunoglobulin G (IgG) solution (Caltag Lab, Burlingame, CA, USA) in PBS for 15 min at 4 °C. Cells were stained (15 min at 4 °C) with fluorescein isothiocyanate (FITC)-labeled cluster of differentiation 4 (CD4) (H129.19) antibody as a specific marker for helper T cells, phycoerythrin (PE)-labeled CD8α antibody as a specific marker for cytotoxic T cells, FITC-labeled CD45R/B220 (RA3-6B2) antibody as a specific marker for pan B cells. All antibodies were purchased from BD Biosciences, unless otherwise indicated. Ten thousand viable cells per mouse (as determined by light scatter profiles) were analyzed using BD FACS Calibur™ flow cytometer (BD Biosciences).

3-2-12. Endogenous hematopoietic colony forming units (CFUs) assay

Next, the ability of SP to rescue and repopulate hemopoietic stem cells in irradiated mice was examined in the spleens to calculate endogenous colony forming units (CFUs).

Spleens were removed from the mice, and their surfaces were examined with the naked eye to score for macroscopic colonies at 9 days after exposure to 7 Gy of irradiation (Zhou et al., 2005).

3-2-13. Hematoxylin and eosin (H&E) staining

To identify whether SP could rescue the intestinal stem cells (crypt cells) from damage induced by gamma ray irradiation, a H&E staining was performed. The small intestines were separated from mice at 9 days after 7 Gy WBI and fixed in 10% buffered formalin. After fixation, the tissues were vertically embedded in paraplast wax to prepare 5 μm sections for H&E staining. As the intestinal crypt microcolony assay technique described by Withers and Elikind (Withers and Elikind, 1970), the number of regenerating crypts per circumference of small intestine section was counted by using a light microscope, and 50 crypt cells per intestinal section were recorded.

3-2-14. Apoptotic fragmentation Assay

To identify the effect of SP on the gamma ray irradiation-triggered apoptosis of jejunal crypt cells, small intestines were separated from mice at 24 h after 2 Gy WBI and fixed in 10% buffered formalin. The tissues were then embedded in paraplast wax to prepare

5 μm sections for H&E staining. Apoptosis was assessed on the morphological evidence of such characteristics as cell shrinkage, chromatin condensation and margination and cellular fragmentation as described by Jee et al. (Jee et al. 2005).

3-2-15. Western blot assay

To evaluate the molecular mechanism by which SP protects radiosensitive cells from gamma ray irradiation-induced apoptosis, the expression patterns of various proteins associated with apoptosis were studied in the small intestine of mice at 1 day after 2 Gy WBI. Tissues lysates from their small intestine were prepared, and cellular protein (40 μg/well) was loaded onto 10-15% SDS-PAGE gels and immunoblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with anti-p53 (1:500, Cell Signaling Technology Inc.), Bax (1:500, Cell Signaling Technology Inc.), Bcl-2 (1:500, Cell Signaling Technology Inc.), and β-actin (1:3000, Sigma), followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology). The blots were developed by enhanced chemiluminescence reagents (iNtRON, Sungnam, Korea) according to the manufacturer's instructions.

3-2-16. Immunohistochemistry (IHC)

For immunohistochemical localization of apoptosis-regulated proteins, tissue sections were incubated with normal horse serum then reacted with antibody to p53 (1:500), Bax (1:500), and Bcl-2 (1:500) for 1 h. The sections were reacted with biotinylated anti-mouse IgG, and with horseradish peroxidase (HRP)-labeled ABC reagent (VECTASTAIN Elite ABC kit; Vector, Burlingame, CA, USA). HRP-binding sites were detected with 3,30-diaminbenzidine (DAB; Vector) and counterstained with hematoxylin.

3-2-17. Animal survival rate

To identify the radioprotective capacity of SP in lethally irradiated mice, the mice were divided into 3 groups as described above. All mice were monitored daily for survival until 30 days after exposure to 9 Gy of gamma radiation. At 31 days after subjected to a single dose of whole body irradiation (WBI), surviving mice were euthanized by cervical dislocation.

3-2-18. Statistical analyses

The results are reported as means ± standard error (S.E.). All results represent three separate experiments. The results were analyzed using the Student's t-test, and $p < 0.05$ was considered statistically significant.

3-3. Results

3-3-1. SP recorded no deaths or hazardous signs in either control or treated groups of mice

In single dose toxicity test, no deaths or hazardous signs were recorded during treatment or the observation period in either control or treated groups of mice and mice receiving oral doses up to 5000 mg/kg of SP by oral route. Also, in sub-acute toxicity test, SP application did not lead to deaths or hazardous signs in doses up to 100 mg/kg by oral route. In addition, the animals of all groups did not show any alteration in general behavior or clinical signs of toxicity.

3-3-2. SP did not affect to the body and organs weight in all groups

As indicated in Table 3-1, in single dose toxicity test, there were no significant differences among the control and treated groups in the body weight gain and the weights of liver, kidney, heart, spleen, and lung during the treatment, although SP 2000 mg/kg-treated group slightly reduced the body weight gain, control group. Also, Table 3-2 showed that SP did not affect to change the weight of body and organs such as liver, kidney, heart, spleen, and lung in all doses (1, 10, and 100 mg/kg) for 21 days.

3-3-3. SP did not show significant difference in hematological parameters among the control and treated groups

As illustrated in Table 3-2, in single dose toxicity test, hematological analysis revealed no difference in parameters among the control and treated groups, although 2000 mg/kg of SP showed a slight difference in WBC. However, all values remained within the reference range. Also, in sub-acute toxicity test, subacute treatment (up to 100 mg/kg) did not cause significant change in most of the parameters (Table 3-4). These results indicated that SP did not have toxicity in mice.

3-3-4. SP protected peripheral blood lymphocytes and splenocytes against DNA damages induced by gamma ray irradiation

The cytoprotective effects of SP against the oxidative DNA damage caused by gamma ray radiation were determined by an alkaline comet assay. Exposure of 2 Gy irradiation (WBI) markedly caused DNA damages as increased tail DNA percentage (%), olive tail movement (μm), and tail length (μm) of peripheral blood lymphocytes, compared to those of the sham irradiated mice (Figure 3-1). In contrast, when SP was administrated with the same dose of WBI, their tail DNA percentage (%), olive tail movement (μm), and tail length (μm) were significantly decreased, respectively. Furthermore, as shown in Figure 3-2, in

splenocytes, DNA damages were considerably caused by gamma ray irradiation, as compared with those of non-irradiated mice. However, SP considerably inhibited DNA damages by decreasing tail DNA percentage (%), olive tail movement (μm), and tail length (μm) comparing to only-irradiated control group (Figure 3-2). These results showed that SP has cytoprotective effects against DNA damages induced by gamma ray irradiation.

Table 3-1. Effect of SP on body and organs weight of mice in single dose toxicity test

Parameter	Control	SP	
		2000 mg/kg	5000 mg/kg
Body weight gain (g)	2.49 ± 0.49	1.392 ± 0.25	1.57 ± 0.45
Liver (g)	0.96 ± 0.76	0.81 ± 0.12	0.93 ± 0.05
Kidney (g)	0.26 ± 0.02	0.25 ± 0.02	0.27 ± 0.03
Heart (g)	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.02
Spleen (g)	0.07 ± 0.00	0.08 ± 0.01	0.07 ± 0.00
Lung (g)	0.16 ± 0.00	0.19 ± 0.03	0.22 ± 0.03

Mean values of 5 animals ± S.E. Control group received saline.

Table 3-2. Hematological parameters in mice after 21 days of treatment with SP in single dose toxicity test

Parameter	Control	SP	
		2000 mg/kg	5000 mg/kg
RBC (10^6/μL)	11.23 ± 0.45	9.89 ± 0.01	10.99 ± 0.00
HGB (g/dL)	16.30 ± 0.30	14.35 ± 0.05	15.80 ± 0.00
HCT (%)	59.35 ± 1.45	51.15 ± 0.05	58.00 ± 0.00
MCV (fL)	52.90 ± 0.80	51.70 ± 0.60	52.80 ± 0.00
MCH (pg)	14.50 ± 0.30	14.50 ± 0.10	14.40 ± 0.00
MCHC (g/dL)	27.45 ± 0.15	28.05 ± 0.45	27.20 ± 0.00
WBC (10^3/μL)	3.00 ± 0.60	1.75 ± 1.05	3.80 ± 0.00

Mean values of 5 animals ± S.E. Control group received saline.

Table 3-3. Effect of SP on body and organs weight of mice in sub acute toxicity test

Parameter	Control	SP		
		1 mg/kg	10 mg/kg	100 mg/kg
Body weight gain (g)	3.23 ± 0.54	2.80 ± 0.29	3.89 ± 0.89	3.53 ± 0.84
Liver (g)	0.91 ± 0.21	0.66 ± 0.34	0.95 ± 0.15	0.87 ± 0.18
Kidney (g)	0.26 ± 0.05	0.24 ± 0.04	0.26 ± 0.02	0.24 ± 0.04
Heart (g)	0.14 ± 0.05	0.14 ± 0.03	0.15 ± 0.03	0.12 ± 0.03
Spleen (g)	0.07± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.08 ± 0.03
Lung (g)	0.15 ± 0.03	0.15 ± 0.02	0.16 ± 0.02	0.22 ± 0.10

Mean values of 5 animals ± S.E. Control group received saline.

Table 3-4. Hematological parameters in mice after 15 days of treatment with SP in sub acute toxicity test

Parameter	Control	SP		
		1 mg/kg	10 mg/kg	100 mg/kg
RBC (10^6/μL)	9.41 ± 0.81	9.57 ± 0.04	9.67 ± 0.74	9.25 ± 0.07
HGB (g/dL)	13.90 ± 1.00	13.85 ± 0.05	14.05 ± 0.95	13.45 ± 0.05
HCT (%)	49.20 ± 3.90	49.45 ± 0.15	48.40 ± 2.60	47.45 ± 0.25
MCV (fL)	52.32 ± 0.35	51.70 ± 0.00	50.15 ± 1.15	51.30 ± 0.10
MCH (pg)	14.80 ± 0.20	14.50 ± 0.00	14.55 ± 0.15	14.55 ± 0.05
MCHC (g/dL)	28.30 ± 0.20	28.00 ± 0.00	29.00 ± 0.40	28.35 ± 0.05
WBC (10^3/μL)	1.85 ± 0.05	1.10 ± 0.20	3.60 ± 1.60	1.55 ± 0.25

Mean values of 5 animals ± S.E. Control group received saline.

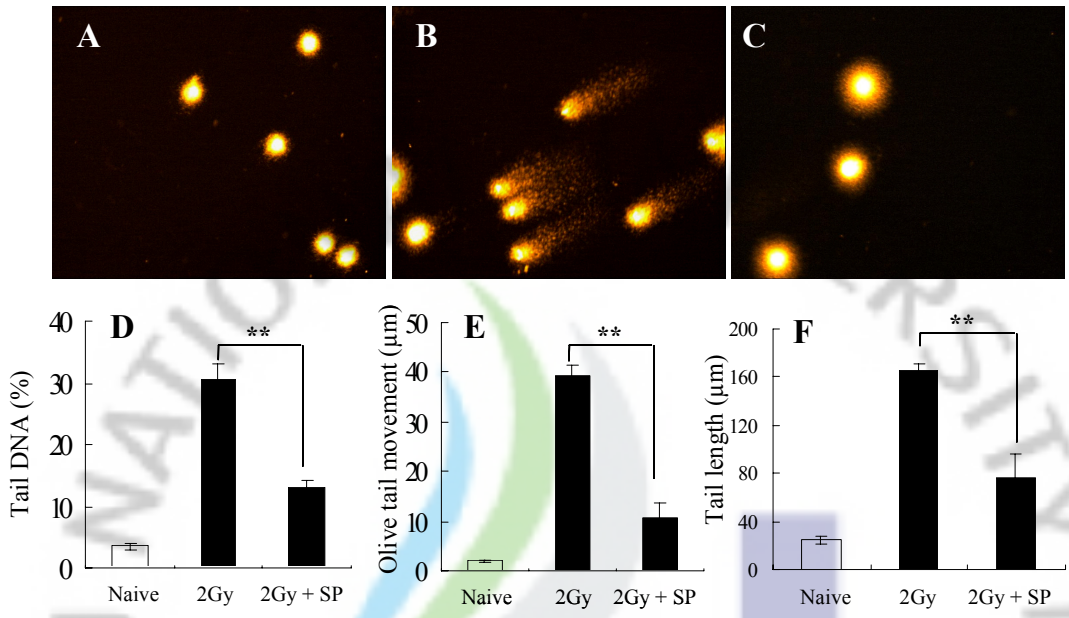

Figure 3-1. Effects of SP on DNA damages in peripheral blood lymphocytes of 2 Gy-irradiated mice. DNA damages in peripheral blood lymphocytes were identified by alkaline comet assay and Komet 5.5 program. (A) Image of cellular DNA damage in peripheral blood lymphocytes of Naïve group, (B) Image of cellular DNA damage in peripheral blood lymphocytes of γ-irradiated group, and (C) Image of cellular DNA damage in peripheral blood lymphocytes of irradiation plus SP-treated. Columns present the tail DNA percentages (D), olive tail DNA movement (E), and Tail DNA length (F) in each group. **, $p < 0.01$.

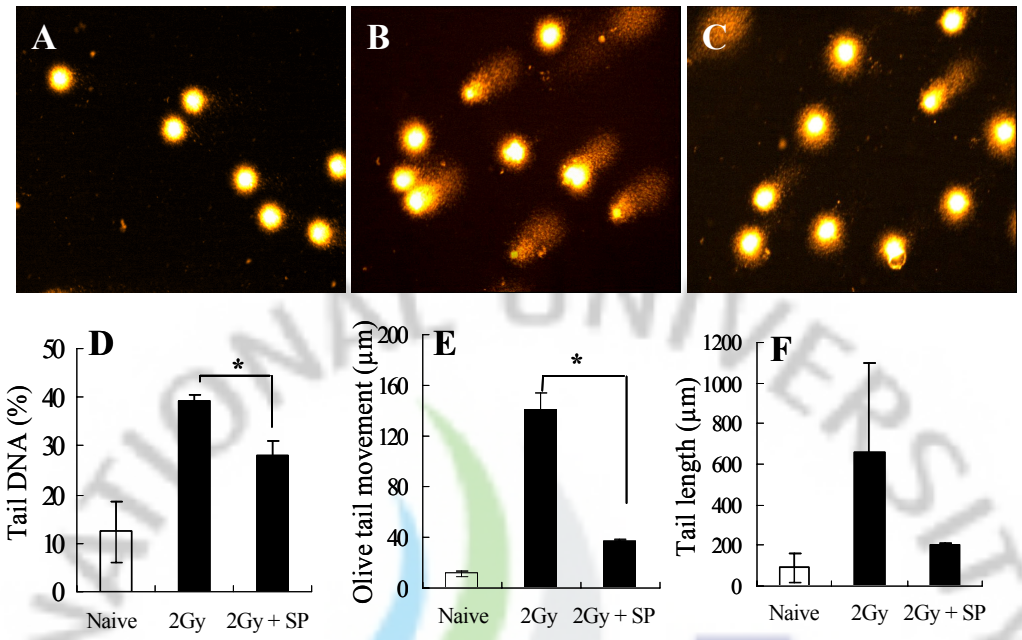

Figure 3-2. Effects of SP on DNA damages in splenocytes of 2 Gy-irradiated mice. The SP dissolved in saline was injected intraperitoneally (i.p.) twice into mice (3 mice/group), receiving the 10 mg/kg b.w. dose first at 18 h and then again at 2 h before irradiation. At 24 h after exposure of 2 Gy irradiation, DNA damages in splenocytes were identified by alkaline comet assay and Komet 5.5 program. (A) Image of cellular DNA damage in splenocytes of Naïve group, (B) Image of cellular DNA damage in splenocytes of g-irradiated group, and (C) Image of cellular DNA damage in splenocytes of irradiation plus SP-treated group. Columns present the tail DNA percentages (D), olive tail DNA movement (E), and tail DNA length (F) of splenocytes in each group. *, $p < 0.05$.

3-3-5. SP stimulated the proliferation of splenocytes in gamma ray irradiated mice

To assess whether SP could stimulate the proliferation and differentiation of splenocytes known as radiosensitive immune cells in gamma ray irradiated mice, the amount of ^{3}H-thymidine incorporated with DNA of splenocytes was measured at 5 days after post-irradiation (2 Gy). As suggested in Figure 3-3, SP significantly elevated the number of splenocytes as much as 2.5-fold above that in only irradiated mice (***; $p < 0.001$). These results indicate that SP improved stimulated the proliferation and differentiation of splenocytes in gamma ray irradiated mice after WBI.

3-3-6. SP improved hemopoiesis in irradiated mice

CFU assay was performed to assess whether SP can rescue and repopulate hemopoietic stem cells in gamma ray-irradiated mice, As Figures 3-4A and B depicts, in the sham irradiated group, there are no colonies in spleens of mice. However, in spleen given sub-lethal doses of gamma irradiation (7 Gy), few endogenous colonies were found (3.8 ± 1.0) and its size was markedly reduced, compared to non-irradiated mice.

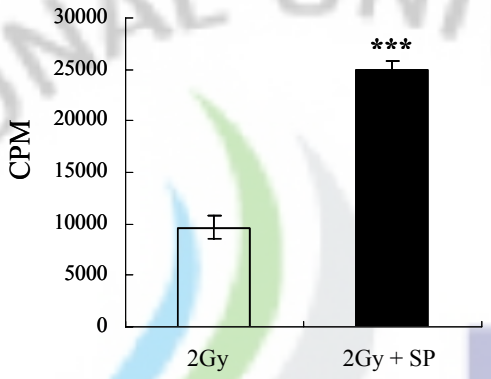

Figure 3-3. Effect of SP on the proliferation of splenocytes in 2 Gy-irradiated mice. At 5 days after exposure of 2 Gy irradiation, the proliferation of splenocytes in mice of each group (3 mice/group) was measured by the incorporation of ^{3}H-thymidine. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. ***, $p < 0.005$.

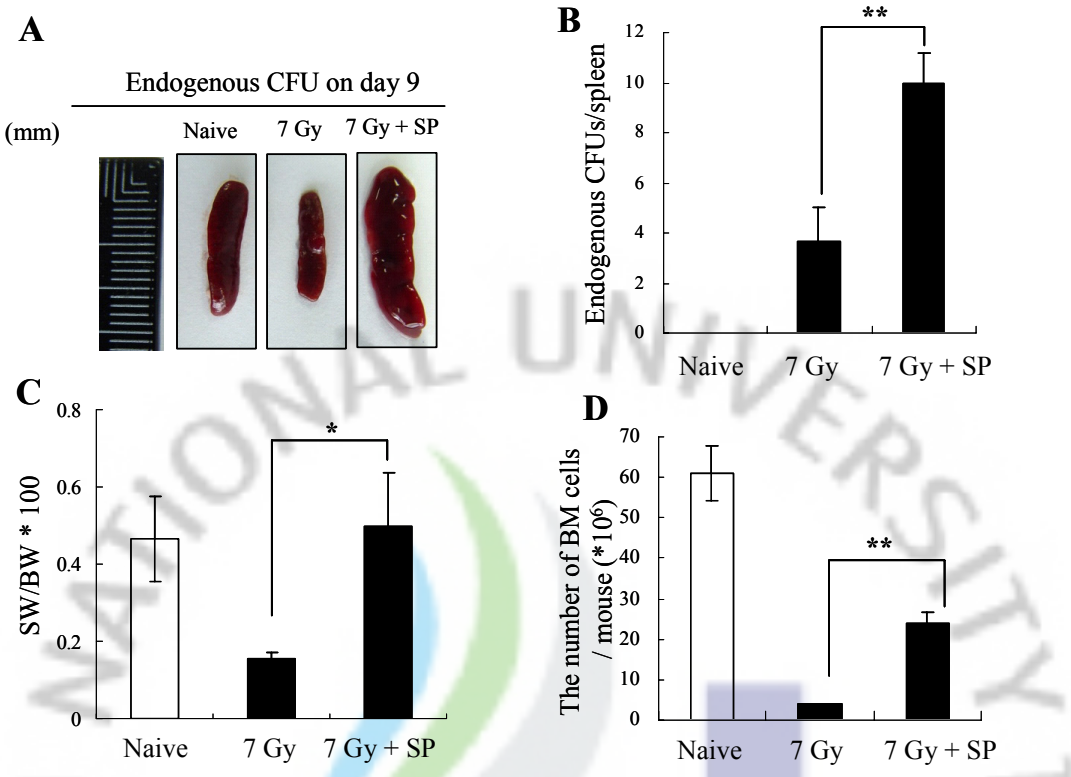

Figure 3-4. Effects of SP on the hematopoietic stem cells in spleen (A, B, and C) and bone marrow (D) in mice exposed to 7 Gy irradiation. (A) Photograph of spleens from non-irradiated, irradiated and SP-treated plus irradiated mice. (B) Columns represent the percentage of CFU in each group. Visible colonies on spleen surface were counted at 9 days after 7 Gy irradiation. (C) Columns represent the percentage of spleen weight on body weight of mice in each group. (D) Columns represent the number of bone marrow cells in each group. Statistical evaluation was performed to compare the experimental groups and corresponding control groups. *, $p < 0.05$, **, $p < 0.01$.

Exposure of gamma ray irradiation decreased the percentage of spleen weight *vs* body weight in mice (Figure 3-4C). In contrast, the treatment of SP increased the size of spleen and significantly the number of endogenous CFUs, as compared with the only gamma ray irradiated mice (9.8 ± 1.6, **; $p < 0.01$). Furthermore, SP significantly increased the percentage of spleen weight *vs* body weight decreased by gamma ray irradiation (about 3 folds, *; $p < 0.05$) (Figure 3-4C).

Next, as shown in Figure 3-4D, exposure of gamma ray (7 Gy) irradiation markedly decreased the number of bone marrow cells in mice as compared with non-irradiated mice. But, the number of bone marrow cells were significantly increased by SP treatment, comparing to only-irradiated mice (about 6 folds, **; $p < 0.01$) (Figure 3-4D)

These results indicate that SP improved the hemopoietic capacities of both spleen and bone marrow in gamma ray irradiated mice

3-3-7. SP enhanced regeneration and survival of intestinal crypts as inhibiting the formation of apoptotic fragments

To define whether SP can rescue the intestinal crypt against damage caused by gamma ray (7 Gy) irradiation, pathological changes was identified in the small intestines from mice of each group. In illustrated in Figure 3-5A, B, D, and E, exposure of gamma ray irradiation

(7 Gy) in mice resulted in severe mucosal damages, evident as marked shortening in the length of villi and their fusion as well as increasing of polymorphism in epithelial cells lining the villi tips, as comparison with those of non-irradiated mice. Additionally, the number of intestinal crypt cells in gamma ray irradiated mice was considerably decreased, compared to those of non-irradiated mice (62.6 ± 3.8 vs 87.6 ± 3.8, Figure 3-5B and D). In sharp contrast, the pretreatment of SP markedly increased not only the length of mucosal villi by their regeneration, but also a significantly number of surviving intestinal crypt cells decreased by gamma ray irradiation in mice (128.4 ± 10.8, Figure 3-5C and F) (*; $p < 0.05$), compared with only irradiated mice.

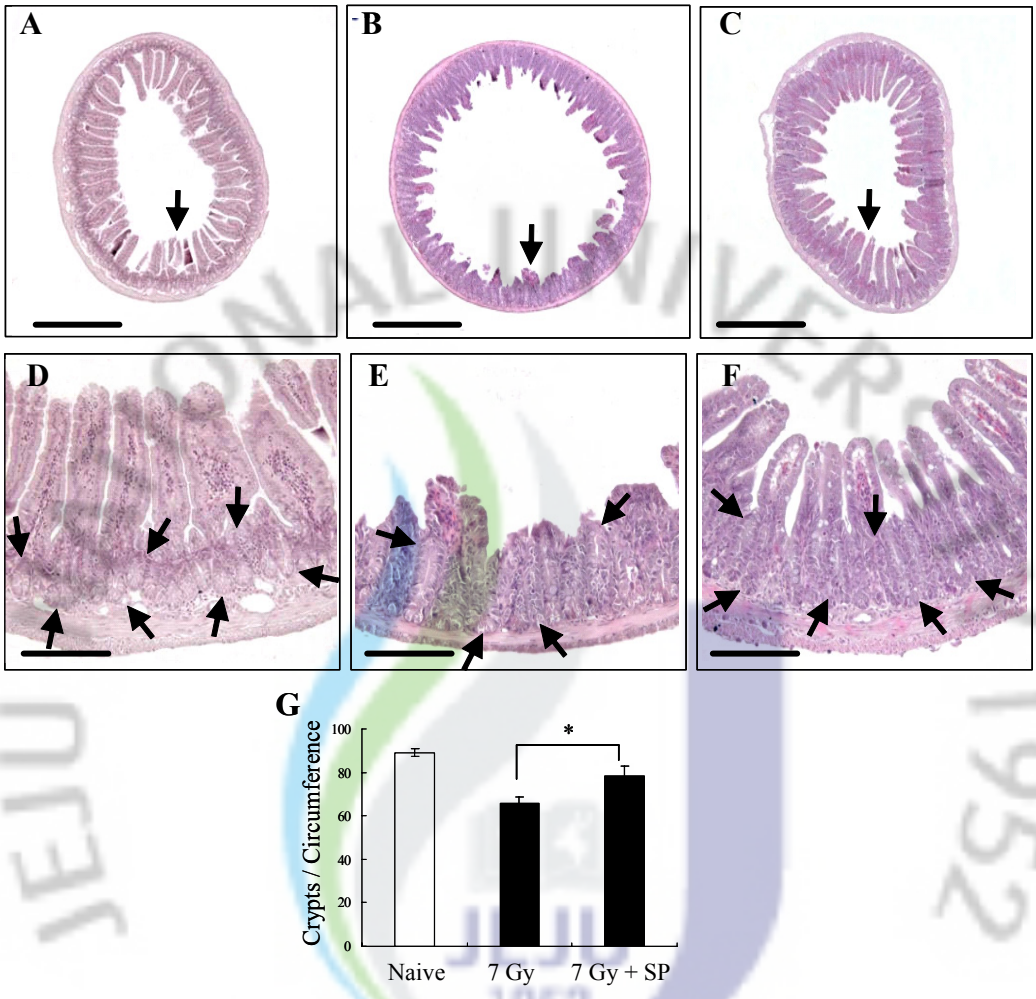

Figure 3-5. Representative images showing villi height and crypt cell survival in the intestinal circumference of sham irradiated (A, D), irradiated controls (B, E) and SP-treated, irradiated mice (C, F). All tissues were collected 9 days after 7 Gy irradiation. (G) Columns indicate the numbers of crypt cells per intestinal circumference (mean ± S.E., *, $p < 0.05$). (A-C) Bars = 300 μm; (D-F) Bars = 60 μm.

Next, the effects of SP on the formation of apoptotic fragments in intestinal crypt cells in gamma ray irradiated mice were investigated. As depicted in Figures 3-6A and C, gamma ray (2 Gy) irradiation caused to markedly increase number of apoptotic fragments in intestinal crypt of mice, comparing to that of non-irradiated mice. In contrast, SP resulted in the significantly marked decrease of apoptotic fragments in intestinal crypt in comparison with that of only irradiated mice (**; $p < 0.01$).

These results suggest that SP enhanced regeneration and survival of intestinal crypt as inhibited the formation of apoptotic fragments in intestinal crypts after gamma ray irradiation.

3-3-8. SP modulated the apoptosis threshold of intestinal crypt cells

To evaluate the molecular mechanism by which SP protects intestinal crypt cells known as radiosensitive stem cells from gamma ray irradiation-induced apoptosis, western blot analysis was used. In gamma ray (2 Gy)-irradiated mice, expression levels of pro-apoptotic molecules such as p53 and Bax were markedly up-regulated, whereas that of anti-apoptotic molecules such as Bcl-2 was slightly decreased, compared with non-irradiated mice (Figure 3-7). However, compared with the gamma ray irradiated mice, SP pretreatment reduced the expression levels of p53 and Bax at 1 day after exposure of gamma ray

irradiation. In addition, the expression level of Bcl-2 was markedly up-regulated by the pretreatment of SP in gamma ray-irradiated mice. β-actin was used for an internal control.

Next, to identify effects of SP on immunohistochemical localization of apoptotic proteins in gamma ray irradiated mice, immunohistochemistry was performed. In this assay, exposure of gamma ray irradiation highly over expressed the immunoreactivities of p53 and Bax, comparing to those of non-irradiated mice (Figures 3-8 and 3-9). Especially, the immunoreactivity of p53 was strongly detected in laminar propria cells in villi core and crypts in gamma ray irradiated mice, in comparison with that of non-irradiated mice, whereas SP pretreatment markedly decreased its immunoreactivities in the same cell types (Figure 3-8).

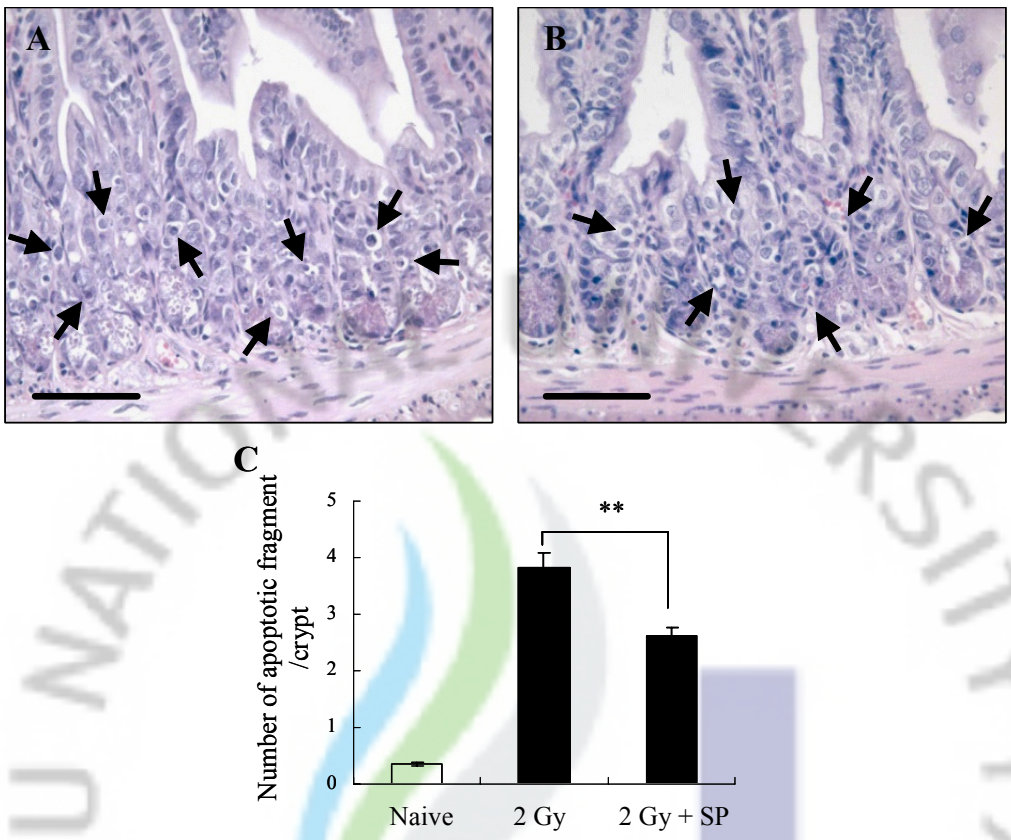

Figure 3-6. Effects of SP on the apoptotic fragmentation of jejunal crypt cells in irradiated controls (A) and SP-treated, irradiated mice (B). The apoptotic fragments of crypt cells show by H&E (arrowheads). (A) 2 Gy irradiation, (B) 2 Gy irradiation plus SP (10 mg/kg) treated, Bars = 30 μm. (C) Columns represent the number of apoptotic fragments per crypt in each group. The mice were sacrificed, and small intestines were obtained 24 h after irradiation. Values are means ± S.E. of 50 crypt sections per 5 small intestine sections from each mouse. (**, $p < 0.01$).

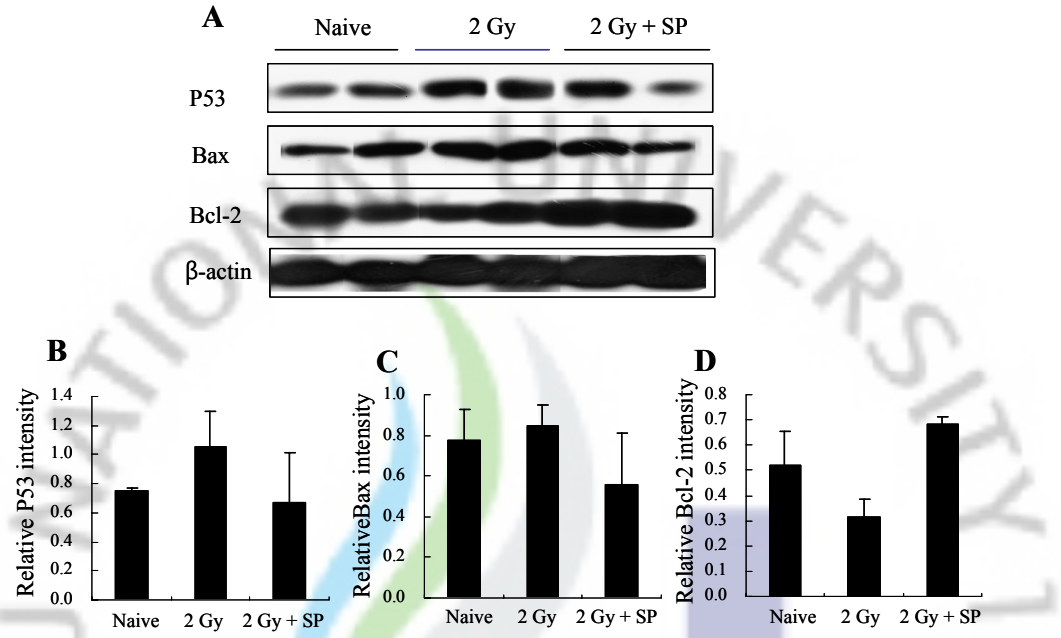

Figure 3-7. Expression of p53, Bax, and Bcl-2 proteins in small intestine of the non irradiated (lanes 1 and 2), 2 Gy irradiated (lanes 3 and 4), and 2 Gy irradiation plus SP (10 mg/kg) treated mice (lanes 5 and 6) by Western blot analysis. The mice (n = 2 per group) were sacrificed, and small intestines were obtained 24 h after irradiation.

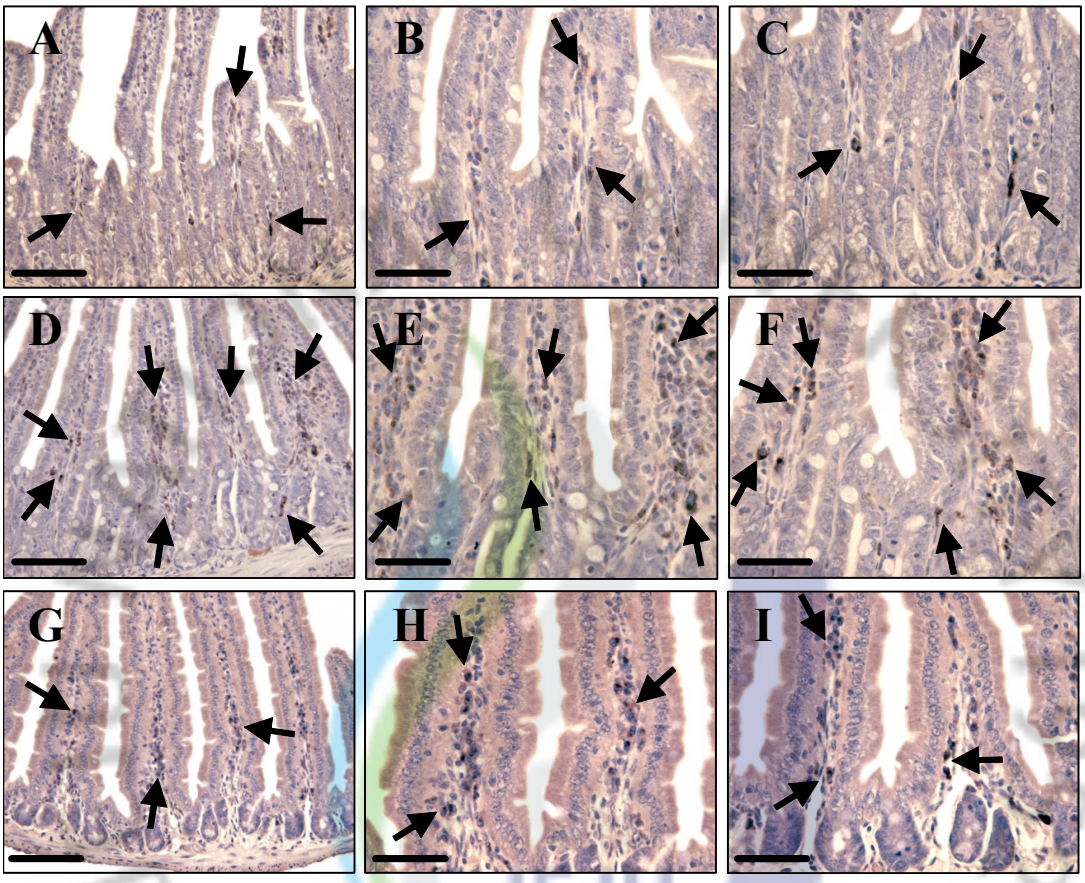

Figure 3-8. The photo presents p53-positive cells with apoptotic fragments evident by immunohistochemistry. (A, B, C) non irradiation, (D, E, F) 2 Gy irradiation, (G, H, I) 2 Gy irradiation plus SP (10 mg/kg) treated, (A, D, G) Bars = 60 μm, (B, C, E, F, H, I) Bars = 30 μm. Mice were sacrificed, and small intestines were obtained 24 h after irradiation.

In addition, Bax protein was highly expressed in epithelial cells of villi and its immunoreactivity was strongly increased by pretreatment of SP after gamma ray irradiation (Figure 3-9). Interestingly, the immunoreactivities of p53 and Bax, two pro-apoptotic proteins in SP treated and irradiated mice showed a similar pattern in non-irradiated mice. Immunosteining. On the other hand, after gamma ray irradiation, Bcl-2 immunoreactivity was weakly detected in laminar propria cells in villi core and crypts, comparing with that of normal mice (Figure 3-10). But, the immunoreactivity of Bcl-2 in laminar propria cells in villi core and crypts was markedly decreased by SP pretreatment and its pattern was similar with normal condition. Moreover, these results showed the similar pattern with those of Western blot assay.

These results revealed that SP improved the hematopoietic efficiency via modulating the apoptosis threshold of intestinal crypt cells in gamma ray irradiated mice

3-3-9. SP enhanced survival of mice after lethal irradiation

To identify the radioprotective capacity of SP in lethally irradiated mice, their survival rates were evaluated for 30 days. First, no deaths occurred in the non-irradiated group for 30 days (data not shown). As illustrated in Figure 3-11 and Table 3-5, the percentage of mice surviving in the only 9 Gy-irradiated group was decreased up to about 0%, compared to a

non-irradiated mice (100%). To the contrary, the survival rate of SP-pretreated mice proceeding 9 Gy-irradiation was markedly improved up to about 60.0% compared with that of the only irradiated mice. Moreover, pretreatment of SP considerably prolonged the survival time of mice decreased by irradiation from 13.5 ± 3.1 to 23.4 ± 10.9 days in comparison with that of the only irradiated mice. These results indicated that the SP induced radio protective efficacy via prolonging the survival of mice against lethally gamma ray irradiation.

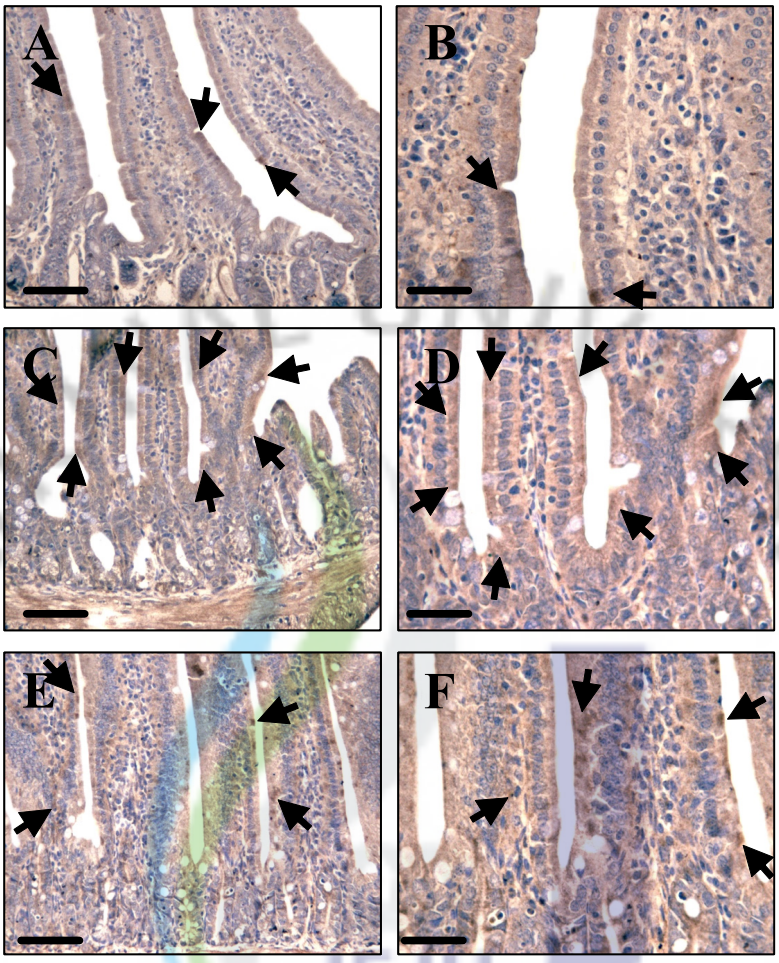

Figure 3-9. The photo presents Bax-positive cells with apoptotic fragments evident by immunohistochemistry. (A, B) non irradiation, (C, D) 2 Gy irradiation, (E, F) 2 Gy irradiation plus SP (10 mg/kg) treated, (A, C, E) Bars = 60 μm, (B, D, F) Bars = 30 μm. Mice were sacrificed, and small intestines were obtained 24 h after irradiation.

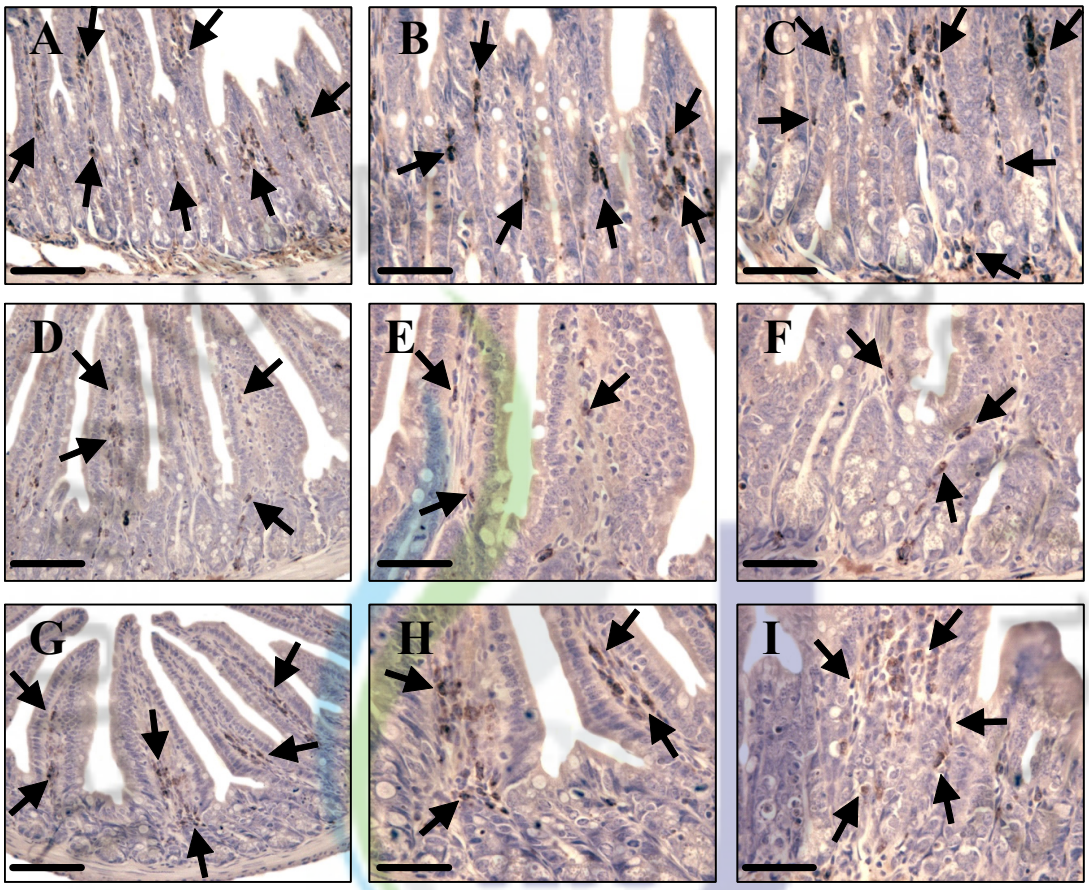

Figure 3-10. The photo presents Bcl-2-positive cells with apoptotic fragments evident by immunohistochemistry. (A, B, C) non irradiation, (D, E, F) 2 Gy irradiation, (G, H, I) 2 Gy irradiation plus SP (10 mg/kg) treated, (A, D, G) Bars = 60 μm, (B, C, E, F, H, I) Bars = 30 μm. The mice were sacrificed, and small intestines were obtained 24 h after irradiation.

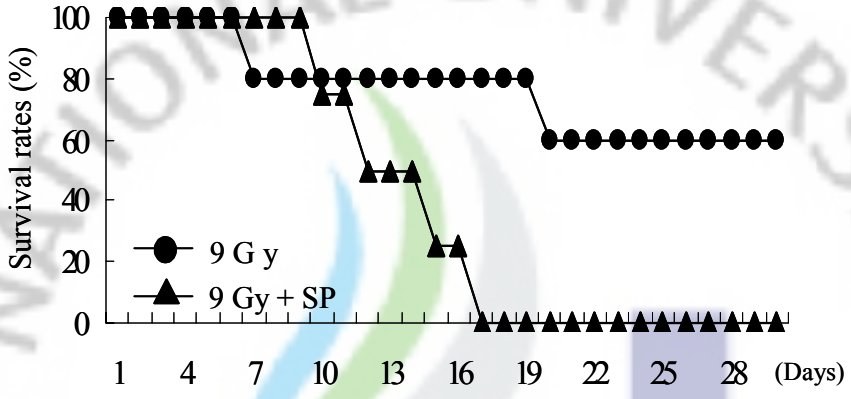

Figure 3-11. The effect of SP on survival of mice exposed to increasing doses of 9 Gy-irradiation. Mice were treated with SP (10 mg/kg b.w.) i.p. at 18 h and 2 h before irradiation. Controls were irradiated but received saline i.p. instead of SP. Results represent five independent experiments. Error bars indicate S.E. of independent experiments.

Table 3-5. The protective effect of SP in lethally gamma ray-irradiated mice

	Survival rate (%)	Mean survival time (days)
9 Gy P.O. (n = 6)	0	13.5 ± 3.1
9 Gy + SP P.O. (n = 6)	60	23.4 ± 10.9

3-4. Discussion

This present study demonstrates that the sulfated polysaccharide (SP) isolated from enzymatic extract of *E. cava*, which is expected as a fucoidan, did not show changes in the body and organs weight and hematological parameters and protected mice from damage caused by gamma ray irradiation.

Normally, the lymphoid and hemopoietic systems are highly sensitive to the exposure of gamma ray irradiation. So, many researchers have tried to search natural products showing radio protective effects as inhibiting the destruction of the lymphoid and hemopoietic systems for development of radio protectors. In addition, previous studies have reported that the production of reactive oxygen species (ROS) generated by radiation induces oxidative stress and plays an important role in cellular damage such as DNA damage, ultimately resulting in cell death (Gandhi et al., 2004). These reports indicate that the destruction of lymphoid and hemopoietic systems is caused by increasing DNA damage followed by the production of ROS induced in gamma ray irradiated mice. Indeed, for few years, previous studies have reported the radio protective effects of polysaccharides and polyphenolic compounds such as eckol, dieckol, phloroglucinol from brown seaweeds are related with their antioxidant effects by exposure of gamma ray irradiation (Park et al., 2008a,b,c, 2009,

and 2010, Moon et al., 2008; .Kang et al., 2006). The previous result also has suggested that SP enhances the survival and proliferation of murine splenocytes as inhibiting the gamma ray irradiation-caused ROS production. In present study, SP protected peripheral blood lymphocytes and splenocytes against DNA damages caused by gamma ray irradiation. The results suggest that the beneficial capacities of SP on the inhibition of ROS production might contribute to the inhibition of DNA damages in lymphoid and hemopoietic systems after exposure of gamma ray irradiation. In addition, it suggested that the inhibitory effects of SP on DNA damages might enhance the proliferation and regeneration of peripheral immune cells or stem cells in the lymphoid and hemopoietic systems in gamma ray irradiation-exposed mice. Indeed, in this experiments, SP enhanced the proliferation of splenocytes and the formation of endogenous splenic colonies (endo CFU) known as an indicator of hematopoiesis which is a critical survival factor as well as the increment of the bone marrow cells. In the ensuing weeks after exposure, a hematologic crisis occurs, characterized by hypoplasia or aplasia of the bone marrow. Therefore, these results suggest that SP improved hemopoiesis by stimulating the hemopoietic and immunostimulatory capacities of both spleen and bone marrow in gamma ray irradiated mice and it was influence to SP's radio protective effects.

Normally, intestinal crypt cells located within the lower part of the epithelial crypts

and possess pluripotency play a central role in mucosal regeneration and can rapidly divide to replenish the number of viable crypts following injury such as gamma ray irradiation (Potten, 1995). However, after radiation, intestinal crypt cells undergone apoptosis can not differentiate quickly enough to repopulate the villi. The result is diminution and blunting of villus height and eventual functional incapacity such as malabsorption, gastrointestinal bleeding and fatal destruction of gastrointestinal tissue, commonly called the GI syndrome (Chen et al., 2007). At these points, many researchers have searched effective and useful substances having the regeneration and survival effects of intestinal crypts that can prevent or rescue of GI injury from radiation exposure. Our results also showed that SP pretreatment enhanced the regeneration and survival of intestinal crypts as inhibiting the formation of apoptotic fragments. In addition, SP inhibited apoptosis in intestine by reducing the expression level and immunoreactivities of p53 and Bax and increasing those of Bcl-2 after exposure of gamma ray irradiation

In previous study, Park et al. have reported that polysaccharide from *Panax ginseng* led to the radio protective effects via inhibiting the p53-dependent pathway which causes the apoptosis of intestinal crypt cells as modulating the expression downstream of Bcl-2, an anti-apoptotic molecule and Bax, pro-apoptotic molecule (Park et al., 2009). Combined, these results suggest that SP improved the regeneration and survival of radiosensitive intestinal

crypt cells seen here by inhibiting radiation-induced apoptosis through the p53-dependent pathway. Interestingly, these radio protective capacities are resistance to opportunistic infections and GI syndrome, which advances the survival of irradiated animals. Indeed, in gamma ray irradiated mice, the treatment of SP enhanced survival of mice. This indicates that the inhibitory efficacy of SP on apoptosis caused in p53-dependent manner resulted in the enhanced survival of mice.

In conclusion, these current results demonstrate that SP did not show toxicities in mice and led to radio protective effects via enhancing immunostimulatory and hematopoietic capacities and inhibiting DNA damages by apoptosis in mice exposed to a lethal dose of WBI. Also, this study proposes that SP can be used as a potential candidate for radiotherapy of cancer patients.

REFERENCES

PART 1

Athukorala, Y., Ahn, G.N., Jee, Y.H., Kim, G.Y., Kim, S.H., Ha, J.H., Kang, J.S., Lee, K.W., Jeon, Y.J., 2009. Antiproliferative activity of sulfated polysaccharides isolated from an enzymatic digest of *Ecklonia cava* on the U-937 cell line. J. Appl. Phycol. 21: 307-314.

Matsuda, M., Yamori, T., Naitoh, M., Okutani, K., 2003. Structural revision of sulfated polysaccharides B-1 isolated from a marine *Pseudomonas species* and its cytotoxic activity against human cancer cell lines. Mar. Biotechnol. 5: 13-19.

Athukorala, Y., Jung, W.K., Vasanthan, T., Jeon, Y.J., 2006. An anticoagulative polysaccharides from an enzymatic hydrolysate of *Ecklonia cava*. Carbohydr. Polym. 66: 184-191.

Teruya, T., Konishi, T., Uechi, S., Tamaki, H., Tako, M., 2007. Antiproliferative activity of oversulfated fucoidan from commercially cultured *Cladosiphon okamuranus TOKIDA* in U-937 cells. Int. J. Biol. Macromol. 41: 221-226.

Cumashi, A., Ushakova, N.A., Preobrazhenskaya, M.E., D'Incecco, A., Piccoli, A., Totani, L., Tinari, N., Morozevich, G.E., Berman, A.E., Bilan, M.I., Usov, A.I., Ustyuzhanina, N.E., Grachev, A.A., Sanderson, C.J., Kelly, M., Rabinovich, G.A., Iacobelli, S., Nifantiev, N.E., 2007. A comparative study of the anti-inflammatory, anticoagulant,

antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. Glycobiology 17 (5): 541-552.

Guzmán, S., Gato, A., Lamela, M., Freire-Garabal, M., Calleja, J.M., 2003. Anti-inflammatory and immunomodulatory activities of polysaccharides from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. Phytother. Res. 17 (6): 665-670.

Sun, H.X., Wang, H., Xu, H., Ni, Y., 2009. Novel polysaccharide adjuvant from the roots of *Actinidia eriantha* with dual Th1 and Th2 potentiating activity. Vaccine 27: 3984-3991.

Nair, P.K., Rodriguez, S., Ramachandran, R., Alamo, A., Melnick, S.J., Escalon, E., Garcia, PI. Jr., Wnuk, S.F., Ramachandran, C., 2004. Immune stimulating properties of a novel polysaccharide medicinal from the plant Tinospora cordifolia. Int. Immunopharmacol. 4: 1645-1659.

Zhu, X.L., Chen, A.F., Lin, Z.B., 2007. *Ganoderma lucidum* polysaccharides enhances the function of immunological effector cells in immunosuppressed mice. J. Ethnopharmacol. 111: 219-226.

Ahn, G., Hwang, I.S., Park, E.J., Kim, J.H., Jeon, Y.J., Jee, Y., 2008. Immunomodulatory effects of an enzymatic extract from *Ecklonia cava* on murine splenocytes. Mar. Biotechnol. 10: 278-289.

Garaude, J., Cherni, S., Kaminski, S., Delepine, E., Chable-Bessia, C., Benkirane, M., Borges, J., Pandiella, A., Iniguez, M.A., Fresno, M., Hipskind, R.A., Villalba, M., 2006. ERK5 activates NF-kappaB in leukemic T cells and is essential for their growth in vivo. J. Immunol.177: 7607-7617.

Garaude, J., Kaminski, S., Cherni, S., Hipskind, R.A., Villalba, M., 2005. The role of ERK5 in T-cell signalling. Scand. J. Immunol. 62: 515-520.

O'Reilly, L.A., Kruse, E.A., Puthalakath, H., Kelly, P.N., Kaufmann, T., Huang, D.C., Strasser, A., 2009. MEK/ERK-mediated phosphorylation of Bim is required to ensure survival of T and B lymphocytes during mitogenic stimulation. J. Immunol. 183 (1): 261-269.

Gerondakis, S., Grumont, R., Rourke, I., Grossmann, M., 1998. The regulation and roles of Rel/NF-κB transcription factors during lymphocyte activation. Curr. Opin. Immunol. 10: 353-359.

Liu, X., Yan, S., Zhou, T., Terada, Y., Erikson, R.L., 2004. The MAP kinase pathway is required for entry into mitosis and cell survival. Oncogene 23 (3): 763-776.

Carretta, M.D., Alarcón, P., Jara, E., Solis, L., Hancke, J.L., Concha, I.I., Hidalgo, M.A., Burgos, R.A., 2009. Andrographolide reduces IL-2 production in T-cells by interfering with NFAT and MAPK activation. Eur. J. Pharmacol. 602: 413-421.

Pimentel-Muiños, F.X., Mazana, J., Fresno, M., 1994. Regulation of interleukin-2 receptor alpha chain expression and nuclear factor. κB activation by protein kinase C in T lymphocytes. Autocrine role of tumor necrosis factor alpha. J. Biol. Chem. 269: 24424-24429.

Armitage, R.J., Alderson, M.R., 1995. B-cell stimulation. Curr. Opin. Immunol. 7 (2): 243-247.

Varga, G., Dreikhausen, U., Kracht, M., Appel, A., Resch, K., Szamel, M., 1999. Molecular mechanisms of T lymphocyte activation: convergence of T cell antigen receptor and IL-1 receptor-induced signaling at the level of IL-2 gene transcription. Int. Immunol. 11: 1851-1862.

Yamamoto, Y., Gaynor, R.B., 2001. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J. Clin. Invest. 107: 135-142.

Prasad, A.S., Bao, B., Beck, F.W., Sarkar, F.H., 2002. Zinc enhances the expression of interleukin-2 and interleukin-2 receptors in HUT-78 cells by way of NF-κB activation. J. Lab. Clin. Med. 140: 272-289.

Granucci, F., Zanoni, I., Feau, S., Ricciardi-Castagnoli, P., 2003. Dendritic cell regulation of immune responses: a new role for interleukin 2 at the intersection of innate and adaptive immunity. EMBO J. 22 (11): 2546-2551.

Mora, A.L., Corn, R.A., Stanic, A.K., Goenka, S., Aronica, M., Stanley, S., Ballard, D.W., Joyce, S., Boothby, M., 2003. Antiapoptotic function of NF-kappaB in T lymphocytes is influenced by their differentiation status: roles of Fas, c-FLIP, and Bcl-xL. Cell Death Differ. 10: 1032-1044.

Dumont, F.J., Staruch, M.J., Fischer, P., DaSilva, C., Camacho, R., 1998. Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. J. Immunol. 160: 2579-2589.

Saito, H., Yamagata, T., Suzuki, S., 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. J. Bio. Chem. 243: 1536-1542.

Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65: 55-63.

Xu, H.S., Wu, Y.W., Xu, S.F., Sun, H.X., Chen, F.Y., Yao, L., 2009. Antitumor and immunomodulatory activity of polysaccharides from the roots of *Actinidia eriantha*. J. Ethnopharmacol. In press.

Sun, H.X., Wang, H., Xu, H.S., Ni, Y., 2009. Novel polysaccharides adjuvant from the roots of *Actinidia eriantha* with dual Th1 and Th2 potentiating activity. Vaccine 27 (30): 3984-3991.

Medeiros, V.P., Queiroz, K.C., Cardoso, M.L., Monteiro, G.R., Oliveira, F.W., Chavante, S.F., Guimaraes, L.A., Rocha, H.A., Leite, E.L., 2008. Sulfated galactofucan from *Lobophora variegata*: anticoagulant and anti-inflammatory properties. Biochemistry (Mosc) 73 (9), 1018-1104.

Hwang, H.J., Kwon, M.J., Kim, I.H., Nam, T.J., 2008. The effect of polysaccharides extracted from the marine alga *Capsosiphon fulvescens* on ethanol administration. Food Chem. Toxicol. 46 (8): 2653-2647.

Shao, B.M., Xu, W., Dai, H., Tu, P., Li, Z., Gao, X.M., 2004. A study on the immune receptors for polysaccharides from the roots of *Astragalus membranaceus*, a Chinese medicinal herb. Biochem. Biophys. Res. Commun. 320 (4): 1103-1111.

Desai, V.R., Ramkrishnan, R., Chintalwar, G.J., Sainis, K.B., 2007. G1-4A, an immunomodulatory polysaccharides from *Tinospora cordifolia*, modulates macrophage responses and protects mice against lipopolysaccharides induced endotoxic shock. Int. Immunopharmacol. 7 (10): 1375-1386.

Schafer, P.H., Gandhi, A.K., Loveland, M.A., Chen, R.S., Man, H.W., Schnetkamp, P.P., Wolbring, G., Govinda, S., Corral, L.G., Payvandi, F., Muller, G.W., Stirling, D.I., 2003. Enhancement of Cytokine Production and AP-1 Transcriptional Activity in T Cells by

Thalidomide-Related Immunomodulatory Drugs. J. Pharmacol. Exp. Ther. 305: 1222-1232.

Angel, P., Karin, M., 1991. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochim. Biophys. Acta 1072 (2-3): 129-157.

Jain, J., Valge-Archer, V.E., Rao, A., 1992. Analysis of the AP-1 sites in the IL-2 promoter. J. Immunol. 148: 1240-1250.

Jain, J., Valge-Archer, V.E., Sinskey, A.J., Rao, A., 1992. The AP-1 site at 2150 bp, but not the NFkB site, is likely to represent the major target of PKC in the IL-2 promoter. J. Exp. Med. 175: 853-862.

Meugge, K., Williams, T.M., Kant, J., Karin, M., Chiu, R., Schmidt, A., Siebenlist, U., Young, H.A., Durum, S.K., 1989. IL-1 costimulatory activity on the IL-2 promoter via AP-1. Science 246: 249-251.

Serfling, E., Barthelma¨s, R., Pfeuffer, I., Schenk, B., Zarius, S., Swoboda, R., Mercurio, F., Karin, M., 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the moue IL-2 gene in T-lymphocytes. EMBO J. 8: 465-473.

PART 2

Kang, K.A., Lee, K.H., Chae, S.W., Koh. Y.S., Yoo, B.S., Kim, J.H., Ham, Y.M., Baik, J.S., Lee, N.H., Hyun, J.W., 2005a. Triphlorethol-A from *Ecklonia cava* protects V79-4 lung fibroblasts against hydrogen peroxide induced cell damage. Free Radic. Res. 39: 883-892.

Kang, K.A., Lee, K.H., Chae, S.W., Zhang, R., Jung, M.S., Lee, Y.G., Kim, S.Y., Kim, H.S., 2005b. Eckol isolated from *Ecklonia cava* attenuates oxidative stress induced cell damage in lung fibroblast cells. FEBS Lett. 579: 6295-6304.

Kang, K.A., Zhang, R., Lee, K.H., Chae, S.W., Kin, B.J., Kwak, Y.S., Park, J.W., Lee, N.H., Hyun, J.W., 2006a. Protective effect of Triphoroethol-A from *Ecklonia cava* gainst ionidimg radiation. J. Radiat. Res. 47: 61-68.

Kang, K.A., Lee, K.H., Chae, S.W., Chae, Zhang, R., Jung, M.S., Ham, Y.M., Baik, J.S., Lee, N.H., Hyun, J.W., 2006b. Cytoprotective effect of phloroglucinol on oxidative stress induced cell damage via catalase activation.. J. Cell. Biochem. 97 (3): 609-620.

Park, E., Ahn, G.N., Lee, N.H., Kim, J.M., Yun, J.S., Hyun, J.W., Jeon, Y.J., Wie, M.B., Lee, Y.J., Park, J.W., Jee, Y., 2008a. Radioprotective properties of eckol against ionizing radiation in mice. FEBS Lett. 582 (6): 925-930.

Park, E., Ahn, G.N., Yun, J.S., Kim, M.J., Bing, S.J., Kim, D.S., Lee, J., Lee, N.H., Park, J.W., Lee, Y.J., Jee, Y., 2010. Dieckol rescues mice from lethal irradiation by accelerating hemopoiesis and curtailing immunosuppression. Int. J. Radiat. Biol. In press.

Park, E., Lee, N.H., Joo, H.G., Jee, Y., 2008b. Modulation of apoptosis of eckol against ionizing radiation in mice. Biochem. Biophys. Res. Commun. 372 (4): 792-797.

Ahn, G.N., Kim, K.N., Cha, S.H., Song, C.B., Lee, J., Heo, M.S., Yeo, I.K., Lee, N.H., Jee, Y., Kim, J.S., Heu, M.S., Jeon, Y.J., 2007. Antioxidant activities of phlorotannins purified from *Ecklonia cava* on free radical scavenging using ESR and H_2O_2-mediated DNA damage. Eur. Food Res. Technol. 226: 71-79.

Kang, H.S., Chung, H.Y., Kim, J.Y., Son, B.W., Jung, H.A., Choi, J.S., 2004. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. Arch. Pharm. Res. 27: 194-198.

Kong, C.S., Kim, J.A., Yoon, N.Y., Kim, S.K., 2009. Induction of apoptosis by phloroglucinol derivative from *Ecklonia Cava* in MCF-7 human breast cancer cells. Food Chem. Toxicol. 47 (7): 1653-1658.

Senni, K., Gueniche, F., Foucault-Bertaud, A., Igondjo-Tchen, S., Fioretti, F., Colliec-Jouault, S., Durand, P., Guezennec, J., Godeau, G., Letourneur, D., 2006. Fucoidan a sulfated

polysaccharide from brown algae is a potent modulator of connective tissue proteolysis. Arch. Biochem. Biophys. 45 (1): 56-64.

Hu, T., Liu, D., Chen, Y., Wu, J., Wang, S., 2010. Antioxidant activity of sulfated polysaccharide fractions extracted from *Undaria pinnitafida* in vitro. Int. J. Biol. Macromol. 46 (2): 193-198.

Wang, J., Zhang, Q., Zhang, Z., Song, H., Li, P., 2010. Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica*. Int. J. Biol. Macromol. 46 (1): 6-12.

Zhuang, C., Itoh, H., Mizuno, T., Ito, H., 1995. Antitumor active fucoidan from the brown seaweed, umitoranoo (*Sargassum thunbergii*). Biosci. Biotechnol. Biochem. 59 (4): 563-567.

Kim, M.H., Byon, Y.Y., Ko, E.J., Song, J.Y., Yun, Y.S., Shin, T., Joo, H.G., 2009a. Immunomodulatory activity of ginsan, a polysaccharide of *Panax ginseng*, on dendritic cells. Korean J. Physiol. Pharmacol. 13 (3): 169-173.

Lim, T.S., Na, K., Choi, E.M., Chung, J.Y., Hwang, J.K., 2004. Immunomodulating activities of polysaccharides isolated from *Panax ginseng*. J. Med. Food. 7 (1): 1-6.

Kim, H.J., Kim, M.H., Byon, Y.Y., Park, J.W., Jee, Y., Joo, H.G., 2007. Radioprotective effects of an acidic polysaccharide of Panax ginseng on bone marrow cells. J. Vet. Sci. 8 (1): 39-44.

Park, E., Hwang, I., Song, J.Y., Jee, Y., 2009. Acidic polysaccharide of Panax ginseng as a defense against small intestinal damage by whole-body gamma irradiation of mice. Acta Histochem. In press.

Lee, T.K., Johnke, R.M., Allison, R.R., O'Brien, K.F., Dobbs, L.J. Jr., 2005. Radioprotective potential of ginseng. Mutagenesis 20 (4): 237-243.

Lee, J., Kim, J., Moon, C., Kim, S.H., Hyun, J.W., Park, J.W., Shin, T., 2008. Radioprotective effects of fucoidan in mice treated with total body irradiation. Phytother. Res. 22 (12): 1677-1681.

Byon, Y.Y., Kim, M.H., Yoo, E.S., Hwang, K.K., Jee, Y., Shin, T., Joo, H.G., 2007. Radioprotective effects of fucoidan on bone marrow cells: improvement of the cell survival and immunoreactivity. J. Vet. Sci. 9 (4): 359-365.

Kim, M.H., Joo, H.G., 2008. Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells. Immunol. Lett. 115 (2): 138-143.

Kang, H.S., Chung, H.Y., Kim, J.Y., Son, B.W., Jung, H.A., Choi, J.S., 2004a. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. Arch. Pharm. Res. 27: 194-198.

Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I., Nakamura, T., 2002. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. J. Antimicrob. Chemother. 50: 889-893.

Fukuyama, Y., Kodama, M., Miura, I., Kinzyo, Z., Kido, M., Nakayama, Y., Takahashi, H., 1989. Structure of an anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome Okamura* and inhibitory activities of its derivatives on plasma plasmin inhibitors. Chem. Pharm. Bull. 37: 349-353.

Lee, J.H., Kim, N.D., Choi, J.S., Kim, Y.J., Moon, Y.H., Lim, S.Y., Park, K.Y., 1998. Inhibitory effects of the methanolic extract of an edible brown alga, *Ecklonia stolonifera* and its component, phlorglucinol on aflatoxin B1 mutagenicity in vitro (Ames test) and on benzo(a)pyrene or *N*-methyl *N*-nitrosourea clastogenicity in vivo (mouse micronucleus test). Nat. Prod. Sci. 4: 105-114.

Ahn, G., Hwang, I., Park, E., Kim, J., Jeon, Y.J., Lee, J., Park, J.W., Jee, Y., 2008a. Immunomodulatory effects of an enzymatic extract from *Ecklonia cava* on murine splenocytes. Mar. Biotechnol (NY). 10 (3): 278-289.

Ahn, G., Park, E., Lee, K.W., Shin, T., Park, J.W., Woo, H.C., Jeon, Y.J., Jee, Y., 2008b. Anti-inflammatory effects of enzymatic extract from *Ecklonia cava* on TPA-induced ear skin edema. Food Sci. Biotechnol. 17 (4): 745-750.

Saito, H., Yamagata, T., Suzuki, S., 1968. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. J. Bio. Chem. 243: 1536-1542.

Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65: 55-63.

Bing, S.J., Kim, M.J., Park, E., Ahn, G., Kim, D.S., Ko, .R.K., Lee, N.H., Shin, T., Park, J.W., Jee, Y., 2010. Modulation of radiation-induced apoptosis by 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG) in murine splenocytes. Biol. Pharm. Bull. In press.

Checker, R., Chatterjee, S., Sharma, D., Gupta, S., Variyar, P., Sharma, A., Poduval, T.B., 2008. Immunomodulatory and radioprotective effects of lignans derived from fresh nutmeg mace (*Myristica fragrans*) in mammalian splenocytes. Int. Immunopharmacol. 8 (5): 661-669.

Subramanian, M., Chintalwar, G.J., Chattopadhyay, S., 2005. Antioxidant and radioprotective properties of an *Ocimum sanctum* polysaccharide. Redox Rep. 10 (5): 257-264.

Ye, J., Li, Y., Teruya, K., Katakura, Y., Ichikawa, A., Eto, H., Hosoi, M., Hosoi, M.,

Nishimoto, S., Shirahata, S., 2005. Enzyme-digested fucoidan extracts derived from seaweed Mozuku of *Cladosiphon novae-caledoniae Kylin* inhibit invasion and angiogenesis of tumor cells. Cytotechnology 47: 117-126.

PART 3

Andreson, R.W., Warner, N.L., 1976. Ionizing radiation and the immune response. Adv. Immunol. 24: 215-335.

Park, E., Ahn, G.N., Lee, N.H., Kim, J.M., Yun, J.S., Hyun, J.W., Jeon, Y.J., Wie, M.B., Lee, Y.J., Park, J.W., Jee, Y., 2008a. Radioprotective properties of eckol against ionizing radiation in mice. FEBS Lett. 582 (6): 925-930.

Park, E., Lee, N.H., Joo, H.G., Jee, Y., 2008b. Modulation of apoptosis of eckol against ionizing radiation in mice. Biochem. Biophys. Res. Commun. 372 (4): 792-797.

Park, E., Hwang, I., Song, J.Y., Jee, Y., 2009. Acidic polysaccharide of Panax ginseng as a defense against small intestinal damage by whole-body gamma irradiation of mice. Acta Histochem. In press.

Park, E., Lee, N.H., Ahn, G.N., Baik, J.S., Lee, J., Hwang, K.K., Park, J.W., Jee, Y., 2008c. Inhibition of Apoptosis by Elaeocarpus sylvestris in Mice Following Whole- body

Exposure to Ionizing Radiation: Implications for Radioprotectors. Food Sci. Biotechnol. 17 (4): 718-722.

Park, E., Ahn, G..N., Yun, J.S., Kim, M.J., Bing, S.J., Kim, D.S., Lee, J., Lee, N.H., Park, J.W., Lee, Y.J., Jee, Y., 2010. Dieckol rescues mice from lethal irradiation by accelerating hemopoiesis and curtailing immunosuppression. Int. J. Radiat. Biol. In press.

Kang, K.A., Zhang, R., Lee, K.H., Chae, S.W., Kin, B.J., Kwak, Y.S., Park, J.W., Lee, N.H., Hyun, J.W., 2006a. Protective effect of Triphoroethol-A from *Ecklonia cava* gainst ionidimg radiation. J. Radiat. Res. 47: 61-68.

Lee, T.K., Johnke, R.M., Allison, R.R., O'Brien, K.F., Dobbs, L.J. Jr., 2005. Radioprotective potential of ginseng. Mutagenesis 20 (4): 237-243.

Kim, H.J., Kim, M.H., Byon, Y.Y., Park, J.W., Jee, Y., Joo, H.G., 2007. Radioprotective effects of an acidic polysaccharide of *Panax ginseng* on bone marrow cells. J. Vet. Sci. 8 (1): 39-44.

Kim, M.H., Byon, Y.Y., Ko, E.J., Song, J.Y., Yun, Y.S., Shin, T., Joo, H.G., 2009a. Immunomodulatory activity of ginsan, a polysaccharide of *panax ginseng*, on dendritic cells. Korean J. Physiol. Pharmacol. 13 (3): 169-173.

Lim, T.S., Na, K., Choi, E.M., Chung, J.Y., Hwang, J.K., 2004. Immunomodulating activities of polysaccharides isolated from *Panax ginseng*. J. Med. Food. 7 (1): 1-6.

Lee, J., Kim, J., Moon, C., Kim, S.H., Hyun, J.W., Park, J.W., Shin, T., 2008. Radioprotective effects of fucoidan in mice treated with total body irradiation. Phytother. Res. 22 (12): 1677-1681.

Byon, Y.Y., Kim, M.H., Yoo, E.S., Hwang, K.K., Jee, Y., Shin, T., Joo, H.G., 2007. Radioprotective effects of fucoidan on bone marrow cells: improvement of the cell survival and immunoreactivity. J. Vet. Sci. 9 (4): 359-365.

Kim, M.H., Joo, H.G., 2008. Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells. Immunol. Lett. 115 (2): 138-143.

Zhou, Y., Mi, M.T., 2005. Genistein stimulates hematopoiesis and increases survival in irradiated mice. J. Radiat. Res. 46: 425-433.

Withers, H.R., Elkind, M.M., 1970. Microcolony Survival Assay for Cells of Mouse Intestinal Mucosa Exposed to Radiation. Int. J. Radiat. Biol. 17 (3): 261-267.

Gandhi, N.M., Maurya, D.K., Salvi, V., Kapoor, S., Mukherjee, T., Nair, C.K., 2004. Radioprotection of DNA by glycyrrhizic acid through scavenging free radicals. J. Radiat. Res. 45 (3): 461-468.

Moon, C., Kim, S.H., Kim, H.C., Hyun, J.W., Lee, N.H., Park, J.W., Shin, T., 2008. Protective Effect of Phlorotannin Components Phloroglucinol and Eckol on Radiation-induced Intestinal Injury in Mice. Phytother. Res. 22: 238-242.

Potten, C.S., 1995. Interleukin-11 protects the clonogenic stem cells in murine small-intestinal crypts from impairment of their reproductive capacity by radiation. Int. J. Cancer. 62 (3): 356-361.

Chen, T., Burke, K.A., Zhan, Y., Wang, X., Shibata, D., Zhao, Y., 2007. IL-2 facilitates both recovery of endogenous hematopoiesis and the engraftment of stem cells after ionizing radiation. Exp. Hematol. 35: 203-213.

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