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Extracellular Signal-Regulated Kinase Is a Major Enzyme in Korean Mistletoe Lectin-Mediated Regulation of Macrophage Functions

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Abstract − Korean mistletoe lectin (KML) is the major component found in *Viscum album var. (coloratum)*, displaying anti-cancer and immunostimulating activities. Even though it has been shown to boost host immune defense mechanisms, the regulatory roles of KML on the functional activation of macrophages have not been fully elucidated. In this study, regulatory mechanism of KML on macrophage-mediated immune responses was examined in terms of KML-mediated signaling event. KML clearly induced mRNA expression of tumor necrosis factor (TNF)-α, the generation of reactive oxygen species (ROS) and phagocytic uptake in RAW264.7 cells. All of these events were strongly suppressed by U0126, whereas TNF-α mRNA was not diminished by SB203580 and SP600125, indicating ERK as a central enzyme managing KML-induced up-regulation of macrophage functions. Indeed, KML strongly induced the phosphorylation of ERK in a time-dependent manner without altering its total level. Therefore, these data suggest that ERK may be a major signaling enzyme with regulatory property toward various KML-mediated macrophage responses.

Keywords: Korean mistletoe lectin, *Viscum album var. (coloratum)*, Macrophage functions, Mitogen-activated protein kinase

INTRODUCTION

Macrophages are one of important antigen-presenting cells with anti-cancer and inflammatory responses. These cells are fully differentiated form of monocytes generated from myeloid progenitors and hematopoietic stem cells. Main functional roles of these cells are known as the removal of cell debris, killing pathogenic microorganisms, and the processing and presentation of antigens ingested by phagocytic (Kinne *et al.,* 2000). It has been generally accepted that the production of numerous pro-inflammatory cytokines [e.g. tumor necrosis factor (TNF)-α and interleukin (IL)-1], chemokines and chemoattractants, and cytotoxic and inflammatory molecules [e.g. nitric oxide (NO), reactive oxygen species (ROS) and prostaglandin (PG)E_2] is a critical event for various immunological responses (Kumar and Jack, 2006; Cho *et al.,* 2008). The stimulation of macrophages by immunogens is tightly linked to the activation of intracellular signaling events such as mitogen activated protein kinases (MAPKs) composed of extracellular signal-regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK), as well as serine/threonine kinases. Due to their significance in innate and adaptive immunity, macrophages are regarded as good target immune cells for the anti-cancer or anti-inflammatory purposes (Michaelsson *et al.,* 1995; Gracie *et al.,* 1999).

Korean mistletoe (*Viscum album* L. var. *coloratum)* is a representative medicinal plant, traditionally used in Korea, Japan and China for sedative, analgesic, anti-spasmolytic, cardiotonic and anticancer purposes (Bishop, 1951). Although numerous studies suggested that Korean mistletoe contains various valuable chemical components such as steroids, triterpens, sesquiterpene lactones, flavonoids and alkaloids (Samuelsson, 1959; Mellstrand and Samuelsson, 1973; Jimenez *et al.,* 2006; Orhan *et al.,* 2006), Korean mistletoe lectins (KML: KML-C, 59.5 kDa) have known to be the most promising principle in this plant. It has been reported to have stronger anti-cancer activity

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than European mistletoe lectins (EML: 60 kDa) against various cancer cell lines (Yoon *et al.,* 1999). In immunological aspects, KML has been reported to positively modulate NK cell activity, antigen-specific antibody production, Th1/Th2 cell activation, and induction of cytolytic T lymphocyte activity and macrophage functions such as NO production (Yoon *et al.,* 2003; Lyu and Park, 2006; Kang *et al.,* 2008). These effects suggest that KML could mediate its immunopharmacological actions with a distinct mechanism from its direct cytotoxicity. Even though the immunomodulatory roles of KML in macrophage-mediated immune responses are being reported, inductive mechanism of KML on macrophage function has not been fully understood so far. In this study, we therefore aimed to prove the modulatory roles of KML on the functional activation of macrophage-like RAW264.7 cells in terms of signaling aspect.

MATERIALS AND METHODS

Materials

FITC-dextran was purchased from Sigma Chemical Co. (St. Louis, MO). U0126, SB203580, and SP600125 were obtained from Calbiochem. (La Jolla, CA). Dihydrorhodamine 123 (DHR123) and dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Molecular Probe (Carlsbad, CA). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264 cells were purchased from the American Tissue Culture Center (Rockville, MD). All other chemicals were of Sigma grade.

Preparation of KML

KML was prepared by a method reported previously (Park *et al.,* 1998; Yoon *et al.,* 1999). Briefly, Korean mistletoe *(Viscum album* L. var. coloratum*)* growing on oak tree was collected in winter in Kangwon province, Korean. KML was purified from the Korean mistletoe by affinity chromatography on asialofetuin-Sepharose 4B as described previously (Park *et al.,* 1998; Yoon *et al.,* 1999). The purity determined by SDS-PAGE and densitometric scanning was more than 98%. The purified KML contained a level of endotoxin below the detection limits (0.0015 EU/ml) as assessed by an Endotoxin assay kit (Sigma, MO, St. Louis).

RT-PCR

For the evaluation of LPS-inducible gene mRNA expression levels, total RNA from KML-treated (or untreated)-RAW264.7 cells was prepared by adding TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase as reported previously (Lee *et al.,* 2009). The primers (Bioneer, Seoul, Korea) used in this experiment are indicated in Table I (F: forward, R: reverse).

Table I. Sequences of the primers for the investigated genes by RT-PCR analysis

Gene		Primer sequences
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCCACGTCGTAGC-3'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

Immunoblotting

Cells (5×10^6 cells/ml) were lysed in lysis buffer (in mM: 20 Tris-HCl, pH 7.4, 2 EDTA, 2 EGTA, 50 β-glycerophosphate, 1 sodium orthovanadate, 1 dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin, 1 benzimide and 2 Phenylmethylsulfonyl fluoride) for 30 min under rotation in a cold-room. The lysates were clarified by centrifugation at 16,000×g for 10 min at 4°C. Soluble cell lysates were immunoblotted and ERK or phospho-ERK were visualized as reported previously.

Determination of phagocytic uptake

To measure the phagocytic activity of RAW264.7 cells, a previously reported method was used with slight modifications (Duperrier *et al.,* 2000). RAW264.7 (5×10^4) cells were resuspended in 100 μl PBS containing 1% human AB serum and incubated with FITC-dextran (0.1 mg/ml) at 37°C and 0°C for 30 min. The incubations were stopped by adding 2 ml ice-cold PBS containing 1% human serum and 0.02% sodium azide. The cells were washed three times with cold PBS-azide and analyzed by flow cytometry.

Determination of ROS generation

The level of intracellular ROS was determined by the change in fluorescence resulting from the oxidation of the fluorescent probe H_2DCFDA or DHR123. Briefly, RAW 264.7 cells (5×10^5 cells/well) were exposed to MAPK inhibitors for 30 min. After incubation, cells were then incubated with KML (40 ng/ml) as an inducer of ROS production at 37°C for 6 h. Cells were incubated with 50 μM H_2DCFDA or 10 μM DHR123 for 1 h at 37°C. The degree of fluorescence, corresponding to intracellular ROS, was determined on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Statistical analysis

A Student's *t*-test and an one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means ± standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. *p* values of 0.05 or less were considered to be statistically significant.

RESULTS AND DISCUSSION

To understand the up-regulatory role of KML in macrophage-mediated immune responses, we first examined its cytotoxic activity on the viability of RAW264.7 cells. KML did not inhibit cell viability of RAW264.7 cells up to 80 ng/ml under 6 h incubation (data not shown). Therefore, we continued our experiments under these concentrations to evaluate its modulatory roles.

Recently, it has been reported that KML is able to modulate various macrophage-mediated immune responses such as cytokine production, phagocytic uptake, and ROS generation (Lee *et al.,* 2007; Kang *et al.,* 2008). Thus, lower concentrations (10 ng/ml) of KML decreased TNF-α expression, but phagocytic uptake was strongly up-regulated at higher concentrations (100 ng/ml) of KML (Lee *et al.,* 2007). Under our conditions (mainly 40 ng/ml of KML), however, we have obtained similar up-regulatory effects on TNF-α production (Fig. 1), phagocytic activity (Fig. 2) and ROS generation (Fig. 3), although the up-regulatory levels by KML were 2-fold less stronger than those by other well-known immunogens such as LPS (data not shown). These data suggest that the KML-mediated immune responses may be variable according to its applicable concentrations. Nonetheless, so far, not many papers regarding the molecular mechanisms on KML-mediated up-

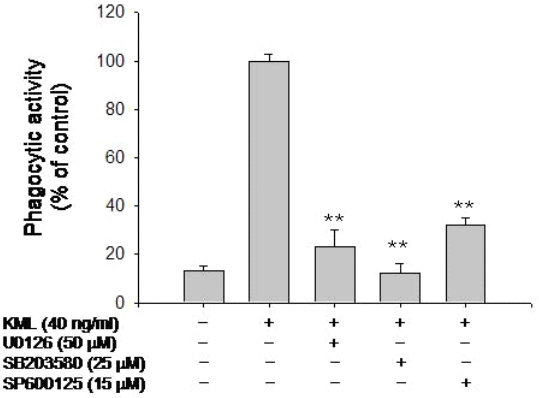

Fig. 2. The effect of MAPK inhibitors on the phagocytic uptake of KML-treated RAW264.7 cells. RAW264.7 cells (2×10^6 cells/ml), pretreated with KML (40 ng/ml), were stimulated with FITC-dextran (1 mg/ml) in the presence or absence of U0126, SB203580, and SP600125 for 6 h. The extent of the phagocytic uptake was determined by flow cytometric analysis, as described in Materials and Methods. Data represent mean ± SEM of three independent observations performed in triplicate. $^{**}p < 0.01$ compared to KML-treated group.

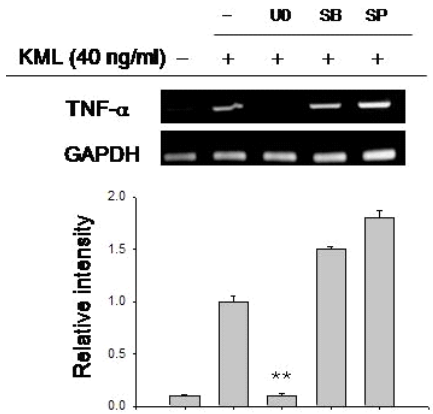

Fig. 1. The effect of MAPK inhibitors on the expression of TNF-α in KML-treated RAW264.7 cells. RAW264.7 cells (1×10^7 cells/ml) were incubated with KML (40 ng/ml) in the presence or absence of U0 (U0126, 50 μM), SB (SB203580, 25 μM), and SP (SP600125, 15 μM) for 6 h. The mRNA level of TNF-α was determined by a semi-quantitative RT-PCR. The results (upper panel) represent one experiment out of three. $^{**}p < 0.01$ compared to KML-treated group.

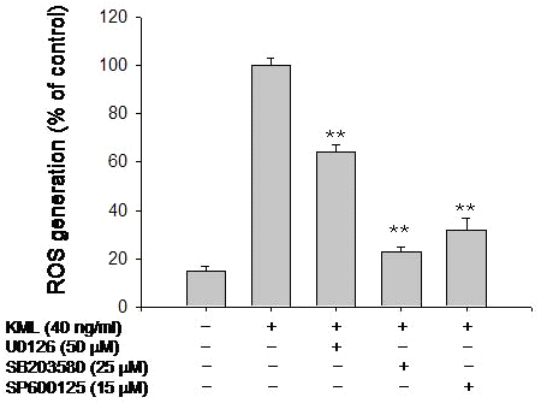

Fig. 3. The effect of MAPK inhibitors on the generation of ROS in KML-treated RAW264.7 cells. RAW264.7 cells (1×10^6 cells/ml) were stimulated with KML (40 ng/ml) in the presence or absence of U0126, SB203580, and SP600125 for 3 h. ROS was determined by flow cytometric analysis as described in Materials and Methods. Data represent mean ± SEM of three independent observations performed in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to KML-treated group.

regulation of macrophage functions have been published. Therefore, we investigated this aspect in terms of KML-induced signaling events. Based on that KML is capable of increasing in the phosphorylation of ERK in RAW264.7 cells, and that KML-induced apoptosis is mediated by activated ERK (Pae *et al.,* 2001; Lee *et al.,* 2007), MAPK inhibitors were employed and tested on their inhibitory effects under TNF-α expression, ROS generation and phagocytosis induced by KML.

Interestingly, U0126, an ERK inhibitor, strongly suppressed all responses, while SP600125, a JNK inhibitor, and SB203580, a p38 inhibitor, did not block TNF-α production but not ROS and phagocytosis, suggesting ERK as the most important signaling component activated by KML. Indeed, it was also found that KML is clearly able to increase the expression of IL-3 (Lee *et al.,* 2007), a cytokine regulating the proliferation of hematopoietic stem cells and their differentiation into macrophages (Wadhwa and Thorpe, 2008). Although effects obtained with other signaling enzyme inhibitors such as the broad-spectrum protein kinase inhibitor, genistein (Geni: 50 μM), a broad-spectrum protein kinase C inhibitor GF109203X (GFX: 10 μM) suggested potential roles of protein tyrosine kinases and protein kinase C in IL-3 production (Lee *et al.,* 2007), MAPKs may play an important role in managing KML-mediated immune responses. Thus, MAPK inhibitors showed inhibitory effects on both ROS generation and phagocytic uptake induced by KML. To confirm the activation of MAPKs, the levels of phospho-MAPKs were examined. As Fig. 4 shows, the enhancement of phospho-ERK level was clearly observed under KML exposure. In contrast, we could not get up-regulation of p38 and JNK, as assessed by their phosphorylation levels, under our conditions (data not shown), suggesting that ERK is the prime target of KML-induced immunostimulating activity. Nonetheless, the inhibitory effects of SB203580 and SP600125 seem to be due to their non-specific inhibition. Otherwise, there might be the activation of p38 and JNK by KML, which could not be detected under our conditions.

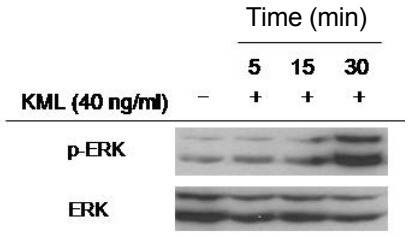

Fig. 4. The effect of KML on the phosphorylation of ERK in RAW264.7 cells. RAW264.7 cells (1×10^7 cells/ml) were incubated with KML (40 ng/ml) for indicated times. The total or phospho-form levels of ERK were determined by Western blotting analysis. The results represent one experiment out of three.

It is not clear yet how KML induces ERK activation and why TNF-α expression, ROS generation and phagocytic uptake are mediated by KML-induced ERK phosphorylation. ERK was reported to be a cellular component participating in KML induction of apoptosis (Pae *et al.,* 2001). It is regarded that ERK-mediated up-regulation of TNF-α by KML seems to be linked to the activation of some transcription factors such as AP-1, since we found that KML never increased NF-κB-mediated reporter gene (luciferase) transcription in HEK293T cells (data not shown). Therefore, evaluation of potential KML-induced transcription factors will be continued in terms of AP-1. It is also considerable that the up-regulation of ROS generation and phagocytic uptake by KML may be connected to its binding to surface galactose-containing proteins (Wu *et al.,* 1992; Lee *et al.,* 2007). Some of these receptor proteins have been reported to be biochemically associated with receptor-mediated endocytosis as well as ROS generating enzyme systems such as NADPH oxidase (Currie *et al.,* 2000; Peterszegi *et al.,* 2003). ERK and p38 are also addressed to play a critical role in receptor-mediated cellular responses such as ROS generation (Parinandi *et al.,* 2003). Therefore, which surface proteins are acting as a KML's target and what kinds of proteins are associated with MAPKs under KML stimulation will be further elucidated.

So far, whether the immunomodulatory activities of KML is much higher than those of other lectins such as European mistletoe lectin (EML) are not clearly evaluated. Direct comparison of their regulatory potency on immune responses seems to be difficult, since there were no experiments examined on the efficacy of lectins at the same time. However, it has been reported that KML more strongly suppresses the proliferation of various tumor cells compared to EML. The pharmacological difference between these lectins seems to be due to their biochemical features originated by distinct N-terminal sequences (Yoon *et al.,* 1999). Thus, KML is composed of four chains (Mr=27.5, 30, 31 and 32.5 kDa) with a broad range of isoelectric points (pI), 8.0 to 9.0, whereas PI values of EML's 2 chains (Mr=29 and 34 kDa) are 6.6-7.0 (Yoon *et al.,* 1999). Although it is clearly defined why differential N-amino acid sequences affect the biochemical characteristics of KML and EML, the differences were found to be tightly linked to the alteration of their apoptosis-inducing potency and sensitivity to D-galactose (Yoon *et al.,* 1999). Therefore, to compare modulatory potency of KML and EML on macro-

phage functions, these lectins should be simultaneously evaluated in terms of phagocytic uptake, ROS generation, cytokine production and NO release from macrophages.

In summary, we have shown that ERK seems to be a major signaling enzyme involved in KML-mediated macrophage activation. Thus, U0126, an ERK inhibitor, strongly suppressed TNF-α expression, phagocytic uptake and ROS generation in KML-treated RAW264.7 cells. In agreement, KML exposure remarkably induced phospho-ERK in a time-dependent manner without altering its total level. Therefore, our data suggest that immunostimulating effect of KML may be mediated by activation of ERK.

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