THE THESIS

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Anti-inflammatory and anti-invasive effects of methanol extract of *Polyopes lancifolius* by suppression of the NFκB pathway

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Chapter I

Methanol Extract of *Polyopes Lancifolius* Inhibits the Expression of Pro-inflammatory Mediators in LPS-stimulated BV2 Microglia Cells via Downregulation of the NF-κB Pathway

Abstract

Purpose: This study aimed to identify the anti-inflammatory and anti-invasive effects of a methanol extract of *Polyopes lancifolius* (MEPL) in lipopolysaccharide (LPS)-stimulated BV2 microglia cells.

Methods: We investigated the expression of mRNA and protein using RT-PCR and western blot analysis in LPS-stimulated BV2 microglial cells. Level of nitric oxide (NO) production was analyzed using Griess reaction. The release of prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) were determined using sandwich ELISA. NF-κB activation was detected using EMSA methods.

Results: We found that MEPL significantly suppressed NO production in LPS-stimulated BV2 cells without any cytotoxicity. Our results also indicated that MEPL decreased the production of PGE_2 and TNF-α in LPS-stimulated BV2 cells. Furthermore, pretreatment with MEPL resulted in a downregulation of LPS-induced mRNA and protein expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-α. Therefore, we investigated the effects of MEPL on nuclear factor-κB (NF-κB) activity, which is a potential transcriptional factor for regulating inflammatory genes such as iNOS, COX-2, and TNF-α. Our data showed that MEPL substantially inhibited the LPS-induced DNA-binding activity of NF-κB. MEPL also suppressed the LPS-induced degradation and phosphorylation of IκBα, and it consequently blocked p65 translocation from the cytosol to the nucleus.

Conclusion: These data show that MEPL may regulate LPS-induced NO, PGE_2, and TNF-α production by suppressing NF-κB activity.

Keywords: Polyopes lancifolius, Nitric oxide, Prostaglandin E_2, tumor necrosis factor-α, Nuclear factor-κB

Introduction

Inflammation is an important host response to external challenge or cellular injury, which is mediated by a variety of cell signaling pathways to balance the restoration of tissue structure and function [1]. As a result of the activation of these signaling pathways, many genes are expressed that produce different kinds of mediators such as nitric oxide (NO) and prostaglandins, which are generated by inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively and cytokines such as tumor necrosis factor-α (TNF-α) [2]. According to recent studies, excessive and abnormal production of these mediators results in severe tissue damage, systemic inflammatory syndrome, septic shock, and atherosclerosis [3]. Therefore, many researchers have recently attempted to identify some food resources and chemicals to suppress the aberrant expression of inflammatory mediators.

Several well-characterized cell signaling pathways involve the production of different cytokines and inflammatory mediators. In particular, the nuclear factor-κB (NF-κB) pathway may play a key role due to rapid activation and its potency as a transcriptional activator [4]. NF-κB regulates several important processes such as cell growth, cancer, apoptosis, inflammation, immune responses, and developmental processes [5]. NF-κB is primarily regulated by its association with inhibitor κB (IκB) proteins in the cytoplasm. NF-κB normally exists in the cytoplasm in an inactive complex bound to IκB [6]. Most agents that activate NF-κB act through a common pathway based on phosphorylation-induced and/or proteasome-mediated degradation of IκB [7]. Removal of the inhibitor can initiate nuclear localization signals on NF-κB subunits. Free NF-κB translocates to the nucleus where it binds to target DNA elements and activates the transcription of genes encoding proteins involved in immune responses, inflammation, or cell proliferation [8]. Therefore, many NF-κB inhibitors, like non-steroidal anti-inflammatory drugs, cell-permeable peptides such as SN-50, and

proteasome inhibitors, have been thought as chemotherapeutic agents, because they suppress a variety of inflammatory diseases [9].

Many seaweed extracts are well-known as regulators of immune and inflammatory responses [10]. Therefore, many researchers are looking for more potent compounds with anti-inflammatory characteristics. *Polyopes lancifolius*, mainly found in the Republic of Korea and Japan, is such a seaweed with medicinal value [11]. Only one study has been conducted and revealed that bromophenol purified from *P. lancifolia,* which was thought to be the same species as *P. lancifolius*, may have potential as a natural nutraceutical for treating type 2 diabetes [11]. Nevertheless, little evidence exists regarding the anti-inflammatory properties of this seaweed.

In this study, we investigated the effects of a methanol extract of *P. lancifolius* (MEPL) on the expression of NO, PGE_2, and TNF-α in lipopolysaccharide (LPS)-stimulated BV2 microglia cells. Our data showed that MEPL downregulated iNOS, COX-2, and TNF-α mRNA expression in LPS-stimulated BV2 cells. MEPL also inhibited LPS-induced NF-κB activation by suppressing the degradation and phosphorylation of IκBα in the cytoplasm and p65 translocation to the nucleus.

Materials and Methods

Preparation of MEPL

MEPL was purchased from Jeju HI-Tech Industry Development Institute (extract NO. 1340; Jeju, Republic of Korea). The red alga, *P. lancifolius* (stock NO. AR038) was collected along the Jeju Island coast of Republic of Korea in April, 2005. Briefly, fresh *P. lancifolius* was washed three times with tap water to remove salt, epiphyte, and sand on the surface of the samples before storage -20ºC. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried powder was extracted with 80% methanol and evaporated *in vacuo*.

Reagents

Lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louise, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). Antibodies against iNOS, COX-2, p65, IκBα, and phospho (p)-IκBα polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against β-actin was from Sigma. Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). Other chemicals were purchased as Sigma grade.

Cell culture and sample treatment

BV2 cell lines were cultured at 37^{o}C in 5% CO_2 in DMEM medium in supplemented with 10% FBS and antibiotics. For the analysis of cell viability, the cells (1×10^5 cells/ml) were incubated with the various concentrations of MEPL 1 h before stimulation of LPS (1.0 μg/ml) for indicated time.

Analysis of cell viability

Cell viability was determined by an MTT assay. BV2 cells (1×10^5 cells/ml) were plated onto 24 well plates and incubated overnight. The cells were pretreated with varying concentrations of MEPL (50, 75, 100, 150, 200 μg/ml) for 1 h and then stimulated with LPS (1.0 μg/ml) for 24 h. MTT assays were used to determine cell viability.

Nitric oxide determination

BV2 cells (1×10^5 cells/ml) were plated onto 24-well plates and pretreated with the indicated concentrations of MEPL 1 h before treatment with LPS (1.0 μg/ml) for 24 h. Supernatants were collected and assayed for NO production using Griess reagent. Nitrite concentration was determined using a dilution of sodium nitrite as a standard.

Measurement of PGE_2 and TNF-α

Expression levels of PGE_2 and TNF-α were measured using enzyme immunosolvent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Isolation of total RNA and RT-PCR

Total RNA was extracted using easy-BLUE^{TM} total RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Two microgram RNA was reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA). cDNA was amplified by PCR using specific primer iNOS (forward 5'-cct cct cca ccc tac caa gt-3' and reverse 5'-cac cca aag tgc ttc agt ca-3'), COX-2 (forward 5'-aag act tgc cag gct gaa ct-3' and

reverse 5'-ctt ctg cag tcc agg ttc aa-3'), TNF-α (forward 5'-gcg acg tgg aac tgg cag aa-3' and

reverse 5'-tcc atg ccg ttg gcc agg ag-3'), and β-actin (forward 5'-tgt gat ggt ggg aay ggg tc-3' and reverse 5'-ttt gat gtc acg cac gat tt-3'). The following PCR conditions were applied: COX-2 and iNOS, 25 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extended at 72°C for 30 s; β-actin, 23 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extended at 72°C for 30 s. β-actin was used as an internal control to evaluate relative expression of iNOS, COX-2, and TNF-α.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction kit (iNtRON Biotechnology; Sungnam, Republic of Korea). The preparation of cytoplasmic and nuclear extracts was conducted using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL, USA). Total cell extracts were separated on polyacrylamide gels and then standard procedures were used to transfer them to the nitrocellulose membranes. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL, USA).

Electrophoretic mobility assay (EMSA)

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary NF-κB binding oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Santa Cruz Biotechnology) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions. Assays were performed using a Lightshift EMSA Optimization kit (Pierce) according to the manufacturer's protocol.

Statistical analysis

All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Marine; Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. All bands were quantified by Scion Imaging software (http://www.scioncorp.com). Statistical analyses were conducted using SigmaPlot software (version 11.0). Values were presented as mean ± SE. Significant differences between the groups were determined using One-Way ANOVA test. Statistical significance was regarded at $P < 0.05$.

Results

Effects of MEPL on cell viability

To determine the effect of MEPL on BV2 cells, an MTT assay was performed at 24 h after treatment with MEPL (50–200 μg/ml) in the presence or absence of LPS. MEPL (50–150 μg/ml) had no cytotoxic effect on BV2 cells. However, cell viability was reduced when 200 μg/ml of MEPL was used with LPS. MEPL as well as LPS (1 μg/ml) alone did not show any cytotoxic effect on BV2 cells (Fig. 1). Therefore, 50–100 μg/ml MEPL was applied in subsequent experiments.

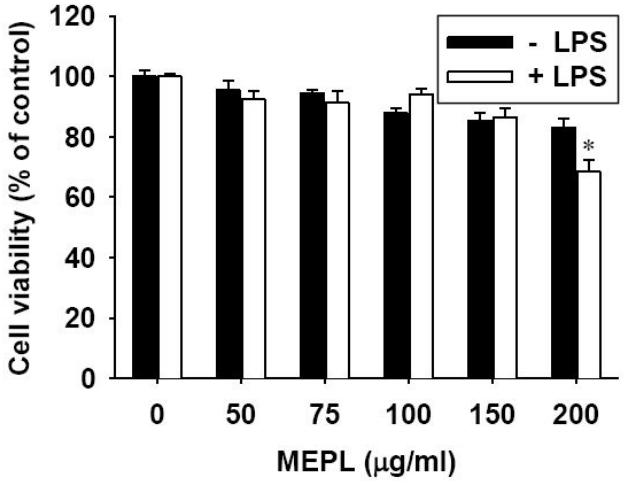

Fig 1: Effects of MEPL on BV2 cell viability (A). Cells (1×10^5 cells/ml) were incubated with the indicated concentrations of MEPL (50–200 μg/ml) for 1 h before lipopolysaccharide (1.0 μg/ml) treatment for 24 h. Cell viability was determined by the MTT assay. Each value indicates mean ± SE and is representative of results obtained from three independent experiments. *$P < 0.05$ was considered significantly different from the value in cells with an untreated control.

Effects of MEPL on LPS-induced NO and PGE_2 production

Cells were stimulated with LPS (1.0 μg/ml) for 24 h after pretreatment with MEPL (100 μg/ml) for 1 h. NO and PGE_2 production was analyzed using the Griess reaction assay and an ELISA, respectively. Stimulating the cells with LPS resulted in a significant increase in NO production (19.3 ± 0.7 μM) compared to the untreated control (5.1 ± 0.2 μM; Fig. 2A). Importantly, MEPL treatment significantly inhibited LPS-induced NO production to the level of the untreated control (5.1 ± 0.2 μM). Consistent with the inhibition of NO production, MEPL treatment (134 ± 11 pg/ml) markedly attenuated LPS-induced PGE_2 production (465 ± 35 pg/ml; Fig. 2B). Our data also revealed that MEPL alone (83 ± 19 pg/ml) sustained PGE_2 production compared to the untreated control (80 ± 21 pg/ml). Taken together, these results indicate that MEPL significantly suppressed the release of NO and PGE_2 in LPS-stimulated BV2 cells.

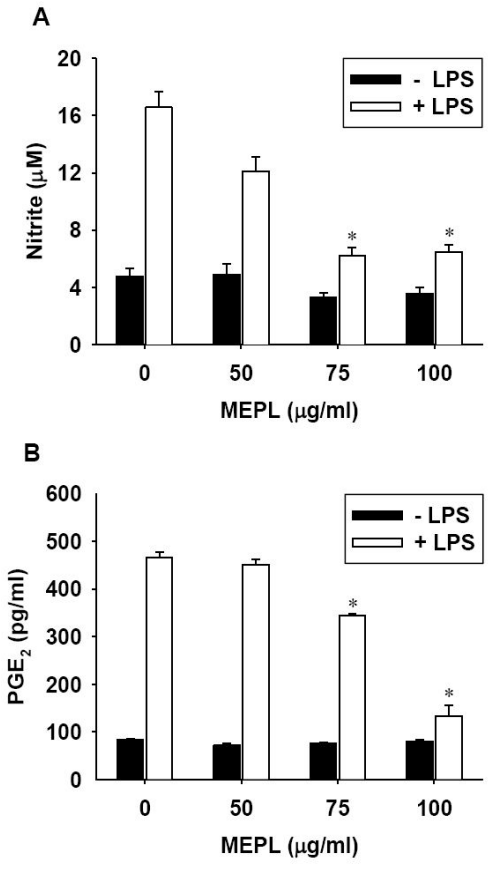

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Fig 2: Effects of MEPL on LPS-induced nitric oxide (NO) (A) and prostaglandin (PG) E_2 (B) production in BV2 cells. Cells (1×10^5 cells/ml) were incubated with 100 μg/ml of MEPL for 1 h before LPS (1.0 μg/ml) treatment for 24 h. (A) The amounts of NO were determined using Griess reagent, and a standard curve was constructed using $NaNO_2$ in culture medium. (B) The levels of PGE_2 in the media were detected using a specific enzyme immunoassay, according to the manufacturer's instruction. Each value indicates mean ± SE and is representative of results obtained from three independent experiments. $*P < 0.05$ was considered significantly different from the value in cells treated with LPS alone.

Effects of MEPL on LPS-induced iNOS and COX-2 protein and mRNA

To access whether MEPL regulates NO and PGE_2 production at the transcriptional level, iNOS and COX-2 protein and mRNA expression were determined by Western blot and RT-PCR analysis, respectively. Although iNOS and COX-2 protein expression increased significantly in the presence of LPS alone, Western blot analysis data showed decreasing iNOS and COX-2 protein expression following the MEPL pretreatment (Fig. 3A). We further investigated these results by conducting an RT-PCR analysis for iNOS and COX-2 mRNA expression. The RT-PCR analysis showed that LPS treatment alone significantly increased iNOS and COX-2 expression at the transcriptional level at 6 h (Fig. 3B). But these expressions were downregulated after pretreatment with MEPL at 100 μg/ml, similar to the Western blot analysis data. Taken together, these data indicate that MEPL suppressed the upregulation of LPS-stimulated iNOS and COX-2 expression at the transcriptional level.

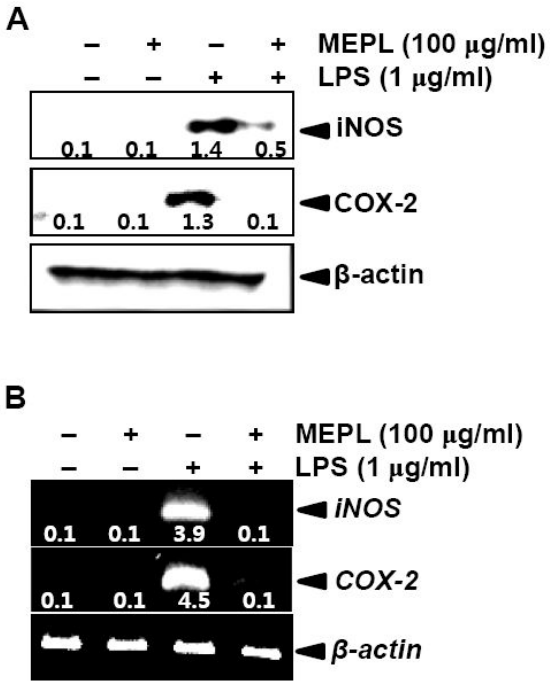

Fig 3: Effects of MEPL on LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expression in BV2 cells. Cells (1×10^5 cells/ml) were incubated with MEPL (100 μg/ml) 1 h before LPS (1.0 μg/ml) treatment for 6 h. (A) Cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against iNOS or COX-2. (B) In a parallel experiment, total RNA was isolated and RT-PCR analyses of iNOS and COX-2 were performed. β-Actin was used as an internal control for the RT-PCR and Western blot analysis. The experiment was repeated three times, and similar results were obtained.

Effects of MEPL on LPS-induced TNF-α production and mRNA expression

Next, we investigated TNF-α and TNF-α mRNA production in LPS-induced BV2 cells. Cells were pretreated with MEPL (100 μg/ml) for 1 h before LPS stimulation for 24 h, and the level of TNF-α in the culture supernatant was determined by ELISA. Consistent with the NO and PGE_2 data, treating the cells with LPS alone significantly increased TNF-α production approximately 10-fold (3533 ± 65 pg/ml) compared to the untreated control (332 ± 39 pg/ml); however, the LPS-induced TNF-α increase was restored to a 50% reduction in the presence of MEPL (1540 ± 35 pg/ml; Fig. 4A). To determine the effect of MEPL on TNF-α gene expression, an RT-PCR analysis was performed at 6 h after LPS treatment. The RT-PCR data showed that MEPL suppressed TNF-α mRNA expression in LPS-stimulated BV2 cells (Fig. 4B). These data indicate that MEPL regulates TNF-α production in LPS-stimulated BV2 microglia cells by regulating TNF-α gene expression.

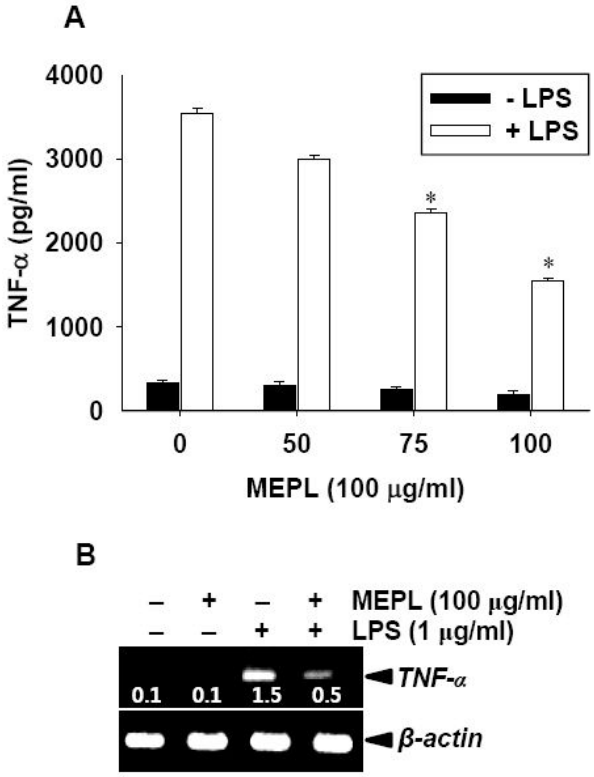

Fig 4: Effects of MEPL on LPS-induced TNF-α production (A) and TNF-α mRNA expression (B) in BV2 cells. Cells (1×10^5 cells/ml) were incubated with 100 μg/ml MEPL for 1 h before LPS (1.0 μg/ml) treatment for the indicated time. (A) After a 24-h incubation, the supernatants were collected, and the amount of TNF-α was measured by ELISA. (B) The level of TNF-α mRNA expression was determined by RT-PCR analysis at 6 h. β-Actin was used as an internal control for the RT-PCR. Each value indicates mean ± SE and is representative of results obtained from three independent experiments. $*P < 0.05$ was considered significantly different from the value in cells treated with LPS alone.

Inhibitory effects of MEPL in LPS-induced NF-κB activity

Activating NF-κB induces the expression of pro-inflammatory mediators such as iNOS, COX-2, and TNF-α [5]. Therefore, we used Western blot analysis and an electrophoretic mobility shift assay (EMSA) assay to investigate how MEPL regulates the specific DNA-binding activity of NF-κB. BV2 cells were preincubated with MEPL for 1 h and then stimulated with LPS for 30 min. The EMSA was conducted to determine whether MEPL inhibits the DNA-binding activity of NF-κB. LPS caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA; however, pretreatment with MEPL significantly reduced LPS-induced NF-κB binding activity (Fig. 5A). In a parallel experiment, LPS significantly induced the phosphorylation and degradation of IκBα and increased p65 expression in the nuclear compartment of BV2 cells (Fig. 5B). However, nuclear translocation of p65, and phosphorylation and degradation of IκBα induced by LPS were inhibited in cells preincubated with MEPL (Fig. 5B). Furthermore, *N*-acetyl-L-cysteine (**NAC**) was used as a potent proteasome and proteases inhibitor of Rel/NF-κB activity to confirm those results. We conducted an RT-PCR analysis to detect iNOS, COX-2, and TNF-α mRNA expression in the presence of NAC. Interestingly, NAC inhibited the expression of LPS-stimulated iNOS, COX-2, and TNF-α expression at the transcriptional level (Fig. 5C). These data indicate that MEPL reduced NF-κB activity in LPS-stimulated BV2 cells by suppressing IκBα phosphorylation and degradation.

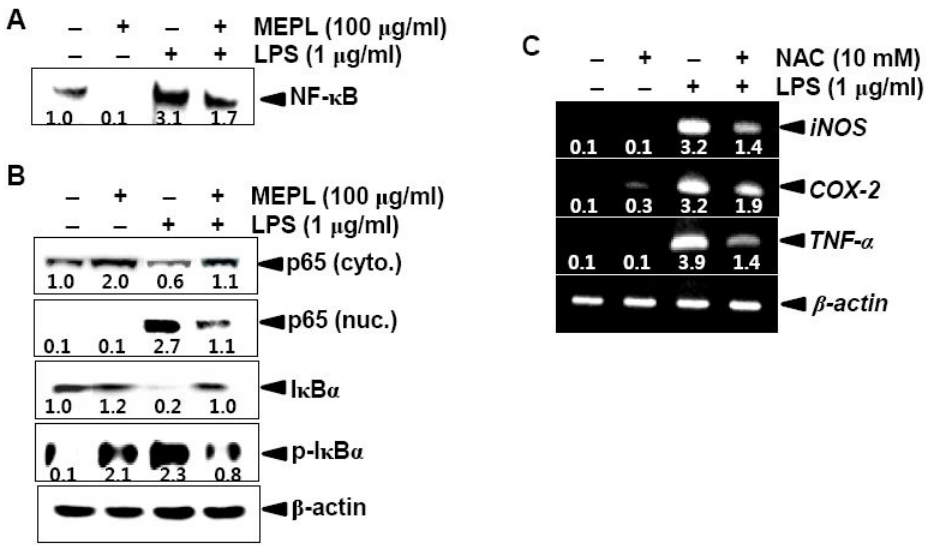

Fig 5: Effects of MEPL on NF-κB DNA binding activity in LPS-stimulated BV2 cells. Cells were preincubated with MEPL (100 μg/ml) for 1 h before stimulation with LPS (1.0 μg/ml) for 30 min. (A) Then the nuclear extracts were assayed for NF-κB activity by electrophoretic mobility shift assay. (B) The nuclear and cytoplasmic extracts were prepared to determine the levels of IκBα and p65 by Western blot analysis. (C) Cells (1×10^5 cells/ml) were incubated with 10 mM *N*-acetyl-L-cysteine (NAC) 1 h before LPS (1.0 μg/ml) treatment for 6 h. Total RNA was isolated, and RT-PCR analyses of iNOS, COX-2, and TNF-α were performed. β-Actin was used as an internal control for RT-PCR. The experiment was repeated three times, and similar results were obtained.

Discussion

Inhibitors of inflammatory mediators and cytokines have been considered an approach to anti-inflammatory drugs. Our data revealed that MEPL inhibited LPS-induced NO and PGE_2 production by suppressing iNOS and COX-2 mRNA expression in BV2 cells without cytotoxicity. MEPL also decreased TNF-α production by suppressing its mRNA expression. Furthermore, we showed that these effects occurred by inhibiting NF-κB activity. These data indicate that MEPL alleviates pro-inflammatory mediators such as NO, PGE_2, and TNF-α via suppression of NF-κB activity.

Expression of NO, PGE_2, and TNF-α plays a critical role in macrophage activation and is also associated with acute and chronic inflammatory diseases (Flanagan et al., 2002). Particularly, NO normally contributes to the control of replication or killing of intracellular microbial pathogens and cancer cells [12]. However, its uncontrolled release can result in inflammatory destruction of target tissues during an infection [13]. The release of iNOS-mediated NO is one of the major factors during inflammatory processes. PGE_2 is another pro-inflammatory mediator involved in inflammatory responses, which is generated by metabolism of arachidonic acid by cyclooxygenase (COX) [14]. Activation of the COX-2 gene is particularly responsible for various inflammatory diseases by inducing PGE_2 overexpression [2]. Accumulating evidence confirms COX-2 as a potential therapeutic target for treating inflammatory diseases [2]. Additionally, TNF-α is a pleiotropic inflammatory cytokine involved in systemic inflammatory diseases [15]. Overproduction of TNF-α switches on signaling pathways that change the cell functions of LPS-stimulated macrophages [16]. As TNF-α plays a role in several diseases, a substantial amount of research has been conducted concerning TNF-α blockers and anti-TNF-α therapies. Recently, anti-TNF-α therapy has been broadly introduced to treat various inflammatory diseases [15].

Therefore, suppressing NO, PGE_2, and TNF-α production by inhibiting their regulatory genes could be a very important therapeutic target for developing anti-inflammatory agents. Our results showed that MEPL attenuated the production of the anti-inflammatory mediators such as NO, PGE_2, and TNF-α in LPS-induced BV2 cells. These data suggest the possibility of developing anti-inflammatory chemicals using MEPL. Nevertheless, further study is needed to elucidate what MEPL component suppresses the expression of anti-inflammatory mediators.

NF-κB has often been referred to as a central mediator and is strongly implicated in variety of inflammatory diseases [9]. Many stimulants including LPS, bacteria, and viruses can lead to NF-κB activation, which leads to the control of the expression of many inflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules [17]. Once NF-κB is fully activated, it participates in the regulation of various target genes in different cells and is involved in different functions [4]. In this study, we showed that MEPL inhibited p65 protein translocation via suppression of IκBα degradation. As a result, MEPL suppressed the LPS-induced DNA-binding activity of NF-κB. Because the expression of many inflammatory genes, iNOS, COX-2, and TNF-α are modulated by NF-κB binding to its specific promoter regions, it is a potential target for suppressing NF-κB activity and regulating LPS-induced inflammation [5]. We tested NF-κB activity with NAC, which is a proteasome and protease inhibitor of Rel/NF-κB activity [18]. According to the RT-PCR analysis, iNOS, COX-2, and TNF-α mRNA expression was suppressed in the presence of NAC. Taken together, these results suggest that MEPL suppresses inflammatory mediators such as NO, PGE_2, and TNF-α via LPS-induced NF-κB activity by suppressing p65 translocation and IκB phosphorylation.

Conclusion

This study showed that MEPL has anti-inflammatory activity, which depended on its ability to regulate NO, PGE_2, and TNF-α production by suppressing NF-κB activation in LPS-stimulated BV2 microglia cells. The data suggest that MEPL has a high potential to treat LPS-induced inflammatory diseases.

Chapter II

Methanol Extract of *Polyopes lancifolius* Suppresses Tumor Necrosis Factor-α-Induced Matrix Metalloproteinase-9 Expression in T24 Bladder Carcinoma Cells

Abstract

Purpose: To investigate the effects of the methanol extract of *Polyopes lancifolius* (MEPL) on the expression of matrix metalloproteinase-9 (MMP-9) and invasion in T24 human bladder carcinoma cells.

Methods: Reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analyses were performed to assess the expression of MMP-9 and its regulatory proteins. MMP-9 activity was evaluated using zymography while matrigel infiltration was performed to assess T24 bladder carcinoma invasion. Electrophoretic mobility assay was used to investigate the nuclear factor-κB (NF-κB) activity.

Results: The expression and activity of MMP-9 were significantly increased in response to TNF-α, but MEPL suppressed TNF-α-induced MMP-9 expression and activity. MEPL also inhibited TNF-α-induced MMP-9 expression at the transcriptional level by blocking the activation of the NF-κB signaling pathway. Furthermore, the extract suppressed TNF-α-induced phosphorylation of IκBα and consequently sustained cytosolic p65 and p50 expression. Matrigel invasion assay showed that MEPL significantly reduced TNF-α-induced invasion of T24 bladder carcinoma cells.

Conclusion: Collectively, these data indicate that MEPL regulates TNF-α-induced MMP-9 expression by suppressing NF-κB activity.

Keywords: *Polyopes lancifolius, Matrix metalloproteinase-9, Invasion; Nuclear factor-κB*

Introduction

Malignant growth of cells in the urinary bladder tissue leads to bladder cancer. Most complications of bladder cancer are attributed to tumor invasion of distant organs, including the regional lymph nodes, lungs, bones, adrenal glands, and intestines [1]. This invasion occurs in multiple steps: tumor cell proliferation, invasion, angiogenesis, intravasation, survival in the circulation, adhesion to endothelial cells, extravasation, and growth in distant organs [2,3].

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are collectively capable of degrading all types of extracellular matrix proteins as well as cleaving cell surface receptors, releasing apoptotic ligands, and activating chemokines and cytokines [4,5]. In particular, MMPs are expressed in nearly all tumors, wherein they facilitate tumor growth, invasion, and metastasis [6]. Among them, MMP-9 is particularly known to play a critical role in bladder cancer progression, including the aspects of angiogenesis, tumor growth, invasion, and distant metastasis. Induction of MMP-9 by growth factors, such as the tumor necrosis factor-α (TNF-α), has been reported to contribute to enhanced cell migration and invasion [7]. In contrast, it has been recently shown that several plant extracts have the ability to decrease MMP-9 secretion and suppress the invasiveness of cancer cells [8]. In addition, a recent study showed that the nuclear factor-kappa B (NF-κB) pathway tightly regulates the expression of MMP-9 in several types of cancer cells [9]. Therefore, NF-κB is considered as a good target to suppress MMP-9 expression in order to inhibit the invasion and metastasis of human bladder cancer.

Polyopes lancifolius is a type of seaweed with medicinal value, which is usually found in the Republic of Korea and Japan [10, 11]. Our previous study established the anti-inflammatory effect of the methanol extract of *P. lancifolius* (MEPL) on lipopolysaccharide

(LPS)-stimulated BV2 microglia cells (unpublished). However, the role of MEPL on the regulation of MMP-9 expression in cancer cells is still unknown. Therefore, in this study, we investigated the ability of MEPL to inhibit MMP-9 expression via suppression of the NF-κB signaling pathway. We also assessed the mechanism by which MEPL regulates the DNA-binding activity of NF-κB and its downstream gene, MMP-9.

Materials and Methods

Preparation of MEPL

MEPL was purchased from Jeju HI-Tech Industry Development Institute (extract no. 1340; Jeju, Republic of Korea). The red alga, *P. lancifolius* (stock no. AR038) was collected along the Jeju Island coast of Republic of Korea in April, 2005. Briefly, fresh *P. lancifolius* was washed three times with tap water to remove salt, epiphyte, and sand on the surface of the samples before storage -20 ºC. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried powder was extracted with 80 % methanol and evaporated *in vacuo*.

Reagents

TNF-α and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co, St Louis, MO, USA. Roswell Park Memorial Institute medium (RPMI) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). Antibodies against p65, p50 and phospho (p)-IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was from Sigma. Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). Other chemicals used were purchased from Sigma.

Cell culture and sample treatment

Human bladder cancer cell line T24 were cultured at 37 ^{o}C in 5 % CO_2 in RPMI medium supplemented with 10 % FBS and antibiotics. For the analysis of cell viability, the cells (1×10^5 cells/ml) were incubated with the various concentrations of MEPL 1 h before stimulation with TNF-α (20 ng/ml) for 24 h.

Cell viability assay

Cell viability was determined by an MTT assay. In brief, T24 bladder carcinoma cells (1×10^5 cells/ml) were plated onto 24 well plates and incubated overnight. The cells were treated with the indicated concentrations of MEPL for 1 h and then stimulated with TNF-α (20 ng/ml). After 24 h, the cells were incubated with a solution of 0.5 mg/ml MTT for 45 min at 37 °C and 5 % CO_2. The supernatant was removed and the formation of formazan was observed by monitoring the signal at 540 nm using a microplate reader.

Isolation of total RNA and RT-PCR

Total RNA was extracted using easy-BLUE^{TM} total RNA extraction kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. Two microgram RNA was reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI). cDNA was amplified by PCR using specific primer MMP-9 (forward 5'-gta ttt gtt caa gga tgg gaa ata c-3' and reverse 5'-gca gga tgt cat agg tca cgt ag-3') and GAPDH (forward 5'-tgt gat ggt ggg aay ggg tc-3' and reverse 5'-ttt gat gtc acg cac gat tt-3'). Reaction products were analyzed on 1.0 % agarose gels, and the bands were visualized by ethidium bromide.

Gelatin substrate gel zymography

The cells were incubated at 37 ^{o}C in 5 % CO_2 in serum free RPMI medium supplemented with 10 % FBS and antibiotics in the absence or presence of MEPL for 24h. The supernatants were collected and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) in 10 % polyacrylamide gels copolymerized with 1 mg/ml of gelatin. After electrophoresis, the gels were washed several times in 2.5 % Triton X-100 for 1 h at room temperature to remove SDS and then incubated for 24 h at 37 °C in reaction buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gels were stained with Coomassie blue

(0.25 %) for 30 min and then destained for 1 h in a solution of acetic acid and methanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Matrigel invasion assay

Invasion assays were performed using Boyden chambers with polycarbonate nucleo pre membrane (Corning, Corning, NY, USA). Cells were trypsinized and 5×10^4 cells were placed onto matrigel-coated transwell for 3 h. The cells were treated with 100 μg/ml of MEPL for 1 h and then stimulated with TNF-α (20 ng/ml). After incubation for 24 h at 37 ^oC in 5 % CO_2, noninvasive cells in upper chamber were removed with a cotton swab. Invaded cells on the lower surface of the filter were fixed and stained with 0.125 % Coomassie blue. Random fields were counted under a light microscope.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction kit (iNtRON Biotechnology; Sungnam, Republic of Korea). Briefly, after treatment with the indicated concentrations of MEPL, cells were harvested, washed once with ice-cold PBS and gently lysed for 15 min in 100 μl ice-cold PRO-PREP lysis buffer. Lysates were centrifuged at 14,000 *g* at 4 °C for 10 min to obtain the supernatants. Supernatants were collected and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA).

Electrophoretic mobility assay (EMSA)

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary

NF-κB (5'-agt tga ggg gac ttt ccc agg c-3') binding oligonucleotides (Santa Cruz Biotechnology) were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions, and annealed for 30 min at room temperature. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5 × Tris borate/EDTA before being transferred onto a positively charged nylon membrane (HybondTM-N+) in 0.5× Tris borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2. Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer's instructions to detect the transferred DNA.

Statistical analysis

All data were derived from at least three independent experiments. Statistical analyses were conducted using SigmaPlot software (version 11.0). Values were presented as mean ± SE. Significant differences between the groups were determined using two-way ANOVA test, statistical significance at $p < 0.05$.

Results

Effect of MEPL on cell viability

To determine the effect of MEPL on T24 bladder carcinoma cell viability, an MTT assay was performed 24 h after treatment with the indicated concentrations (50−200 μg/ml) of MEPL in the absence or presence of TNF-α (20 ng/ml). In the range of 25−150 μg/ml MEPL showed no cytotoxicity in T24 bladder carcinoma cells. However, the viability of the cells was reduced to approximately 80%, when 200 μg/ml MEPL was used (Fig 1). DMSO (0.1 %) as a solvent (data not shown) in the presence of TNF-α (20 ng/ml) did not produce any cytotoxic effects on the viability of T24 bladder carcinoma cells. Therefore, a 100 μg/ml concentration of MEPL was used in the remaining experiments.

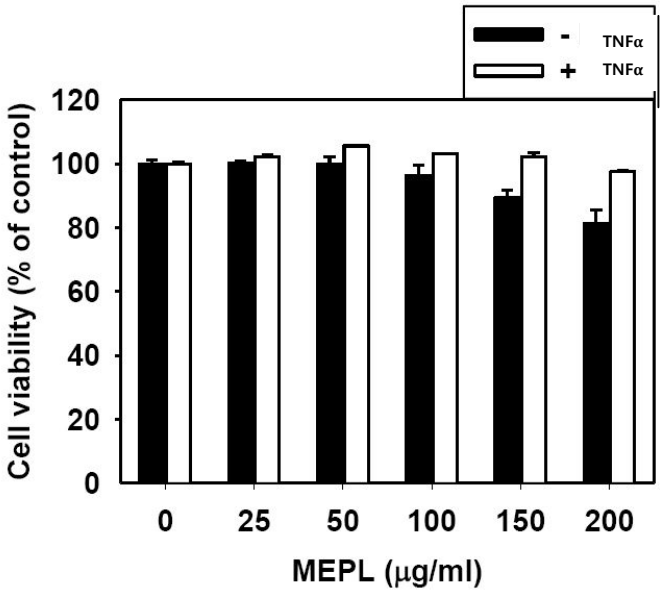

Fig 1: Effect of MEPL on the viability of T24 bladder carcinoma cells. Cells (1×10^5 cells/ml) were incubated with the indicated concentrations of MEHC (50–200 μg/ml) 1 h before TNF-α (20 ng/ml) treatment for 24 h. Cell viability was determined by MTT assay; data are mean ± SE, n = 3; *$p < 0.05$

Suppression of MMP-9 gene transcription and its activity by MEPL

In order to assess whether MEPL decreases the expression of MMP-9, zymography, Western blot analysis and RT-PCR were performed. Zymography data showed that MEPL suppresses

the expression of MMP-9 activity compared to the TNF-α-stimulated group (Fig 2A). Although MMP-9 protein expression increased significantly in the presence of only TNF-α, Western blot analysis showed decreased MMP-9 protein expression following MEPL pretreatment (Fig 2B).

To assess whether MMP-9 gene expression is regulated by MEPL, RT-PCR analysis was conducted; the results showed that 6 h of TNF-α treatment significantly increased MMP-9 expression at the transcriptional level (Fig 2C). Furthermore, MMP-9 gene expression was downregulated after pretreatment for 1 h with 100 μg/ml MEPL, which was similar to the results obtained by zymography and Western blot analysis. Taken together, these data indicate that MEPL suppresses upregulation of TNF-α-induced MMP-9 expression at the transcriptional level.

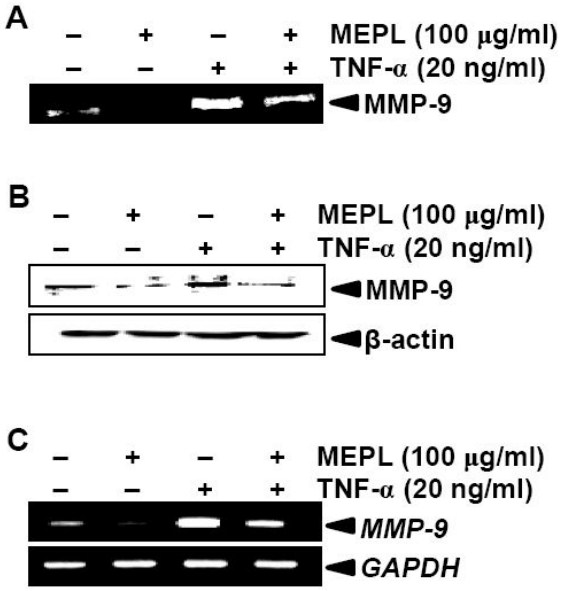

Fig 2: Effect of MEPL on TNF-α-induced MMP-9 protein and mRNA expression in T24 bladder carcinoma cells. (A) Cells were treated with 100 μg/ml MEPL 1 h before TNF-α (20 ng/ml) treatment for 24 h. Conditional medium was collected after 24 h, followed by gelatin zymography. (B) Equal amounts of cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against MMP-9. (C) In a parallel experiment, cells (2×10^5 cells/ml) were incubated with the indicated concentrations of MEPL 1 h before TNF-α (20 ng/ml) treatment for 6 h. Total RNA was isolated and RT-PCR was performed using MMP-9 specific primers. ***Note:*** GAPDH and β-actin were used as internal controls for RT-PCR and Western blot analyses, respectively. The experiments were repeated three times, and similar results were obtained.

Inhibitory effect of MEPL on TNF-α-induced NF-κB activity

NF-κB signal pathway is known to play a critical role in modulating MMP-9 expression [12]. The EMSA data showed that TNF-α caused a significant increase in the amount of binding complexes between NF-κB and specific-binding DNA. Pretreatment with MEPL, however, significantly reduced TNF-α-induced NF-κB activity in the T24 bladder carcinoma cells (Fig 3A). In a parallel experiment, TNF-α significantly induced phosphorylation of IκBα and decreased p65 and p50 expression in the cytosol (Fig 3B). However, MEPL sustained p65 and p50 expression and blocked phosphorylation of IκBα in response to TNF-α. These data indicate that MEPL reduces NF-κB activity in TNF-α-stimulated T24 bladder carcinoma cells by suppressing IκBα phosphorylation.

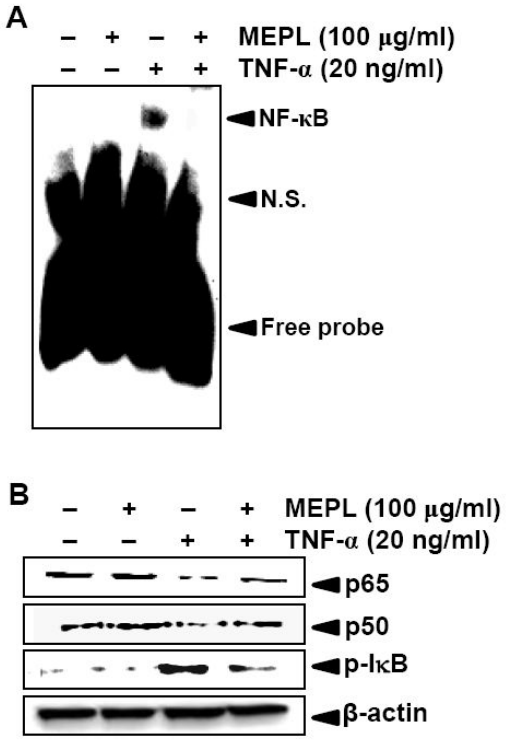

Fig 3: Effect of MEPL on NF-κB DNA binding activity in T24 bladder carcinoma cells. Cells were preincubated with MEPL (100 μg/ml) 1 h before stimulation with TNF-α (20 ng/ml) for 30 min. (A) The nuclear extracts were assayed for NF-κB activity by EMSA and (B) the expression or phosphorylation levels of p65, p50 and p-IκBα was analyzed using Western blot analysis. ***Note:*** β-Actin was used as an internal control for western blot analyses. The experiment was repeated three times and similar results were obtained' N.S. = non-specific

Reduction of invasion by MEPL in T24 bladder carcinoma cells

MMP-9 is thought to be critically involved in the process of tumor invasion [8]. The results of the matrigel assay showed that invasion of T24 bladder carcinoma cells sharply increased, when cells were treated with 20 ng/ml TNF-α (Fig 4A). However, treatment with 100 μg/ml MEPL 1 h prior to TNF-α treatment significantly reduced the invasion of T24 bladder carcinoma cells to approximately 50 %, as compared to that in the TNF-α-treated group (Fig 4B). These results indicate that treatment with MEPL inhibits TNF-α-induced invasion of T24 bladder carcinoma cells.

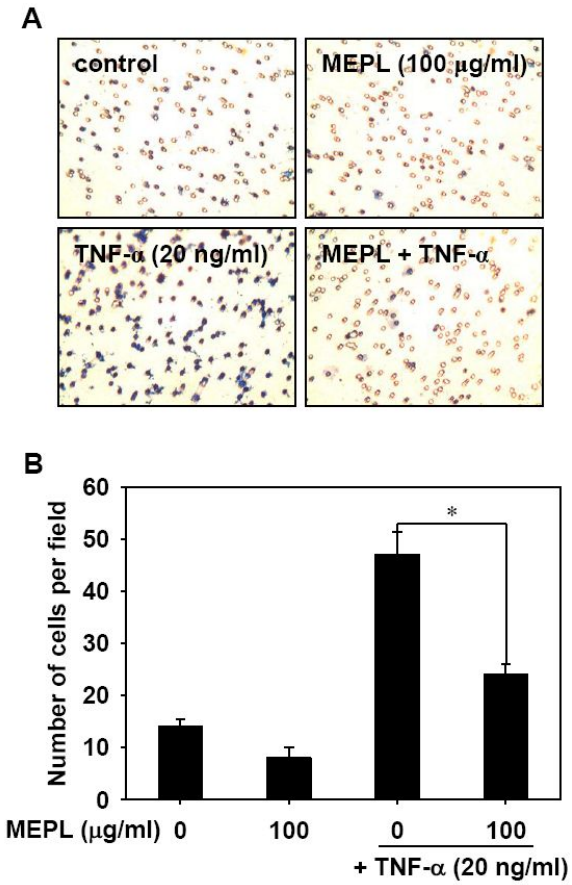

Fig 4: Inhibitory effect of MEPL invasion of T24 bladder carcinoma cells. (A) The upper compartments of Transwells were coated with matrigel for the invasion assay; (B) data are mean ± SE, n = 3; the number of cells per field were estimated.

Discussion

We previously reported that MEPL has anti-inflammatory effect on LPS-stimulated BV2 microglia cells (unpublished data). However, the mechanism by which MEPL inhibits invasion of tumor cells has not been elucidated completely. Therefore, in this study, we evaluated the effects of MEPL on the invasion of T24 bladder carcinoma cells. Our study is the first to provide evidence that MEPL inhibits TNF-α-induced MMP-9 expression in T24 bladder carcinoma cells by blocking NF-κB activation.

In the process of cancer invasion, cells can escape from the primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow into distant foci in normal tissues elsewhere in the body [13]. Cancer cells are involved in numerous interactions with the extracellular matrix and its proteins such as growth factors and cytokines. MMPs are thought to be critical molecules that assist cancer cells during invasion. MMP-9, in particular, is regarded as the main molecule involved in the malignant progression to tumor invasion [14]. MMP-9 activation is specifically associated with tumor progression and invasion in bladder tumors [15]. Therefore, inhibition of MMP-9 expression is a strategic target for the development of a therapeutic experimental model of tumor invasion and metastasis. As shown by the matrigel assay, MEPL significantly suppressed cell invasion. According to the zymography data, MEPL treatment resulted in a decrease of MMP-9 activity in the culture medium. These data support the assertion that MEPL is a potential natural resource that can inhibit cancer cell invasion via suppression of MMP-9 expression.

Expression of the transcription factor NF-κB is induced by various distinct stimuli. Activated NF-κB, in turn, modulates transcription of many target genes including MMP-9 [16]. NF-κB is a ubiquitous transcription factor that responds rapidly in mammalian cells and is strongly activated by the cytokines interleukin-1 and TNF-α under various pathological

conditions [17,18]. In nonstimulated cells, NF-κB is present in the cytosol where it is complexed with its inhibitor IκB. Activation of NF-κB depends on the phosphorylation of IκB induced by a signal of a specific IκB kinase, which initiates conjugation of the inhibitor's with ubiquitin and subsequent phosphorylation/degradation [19]. In this study, we showed that MEPL inhibits TNF-α-induced DNA-binding.

CONCLUSION

These results obtained in this study support the fact that MEPL is a tactic regulator that inhibits MMP-9 expression via suppression of NF-κB activity. We confirmed that MEPL downregulates the expression of MMP-9 activity by suppressing TNF-α-induced activation of NF-κB. Therefore, MEPL may be an effective therapeutic source for regulating tumor invasion.

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