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Research Article

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

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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*

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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.

EXPERIMENTAL

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 °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™ 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 °C in 5 % CO_2 in serum free RPMI medium supplemented with 10 % FBS and antibiotics in the absence or presence of MEPL for 24 h. 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 5mM $CaCl_2$ and 1μM $ZnCl_2$. 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 °C 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 chemilu-

minescence detection system (model RPN2108, 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, with statistical significance set 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.

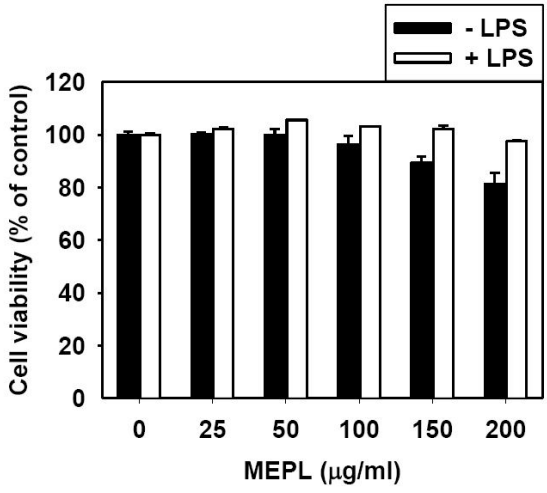

Fig 1: Effects of MEPL on the viability of T24 bladder carcinoma cells. Cells (1 × 10^5 cells/ml) were incubated with the indicated concentrations of MEHC (50–200 µg/ml) 1 h before TNF-α (20 ng/ml) treatment for 24 h. Cell viability was determined by MTT assay; data are mean ± SE, n = 3; *$p < 0.05$

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.

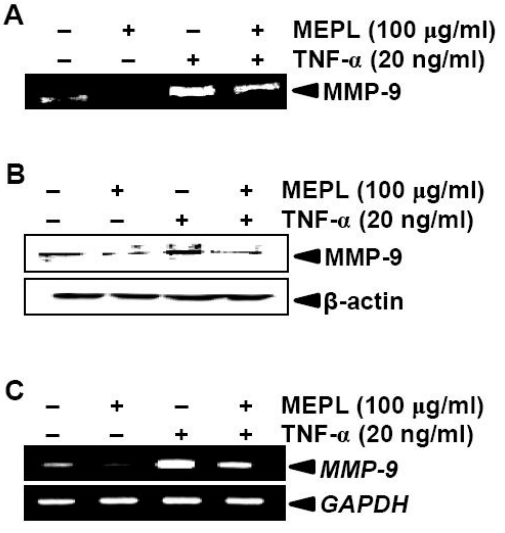

Fig 2: Effects of MEPL on TNF-α-induced MMP-9 protein and mRNA expression in T24 bladder carcinoma cells. (A) Cells were treated with 100 µg/ml MEPL 1 h before TNF-α (20 ng/ml) treatment for 24 h. Conditional medium was collected after 24 h, followed by gelatin zymography. (B) Equal amounts of cell lysates 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

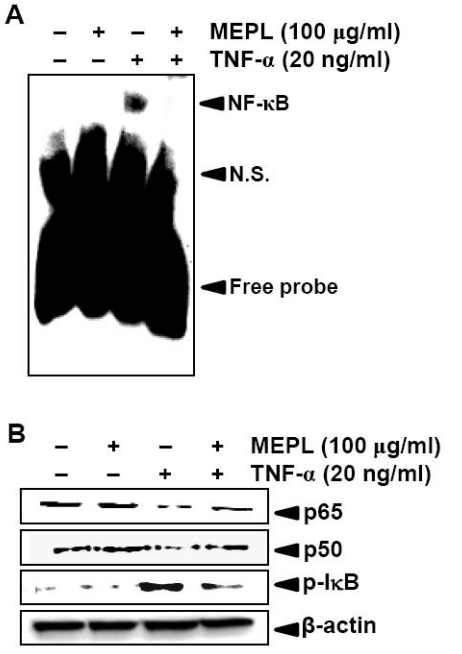

Fig 3: Effect of MEPL on NF-κB DNA binding activity in T24 bladder carcinoma cells Cells were preincubated with MEPL (100 µg/ml) 1 h before stimulation with TNF-α (20 ng/ml) for 30 min. (A) The nuclear extracts were assayed for NF-κB activity by EMSA and (B) the expression or phosphorylation levels of 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.

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.

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.

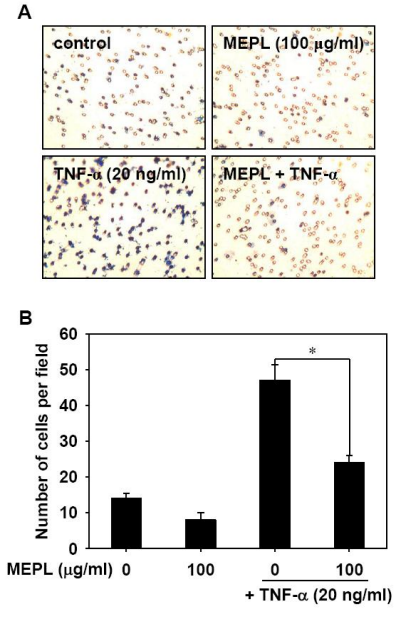

Fig 4: Inhibitory effect of MEPL invasion of T24 bladder carcinoma cells. (A) The upper compartments of Transwells were coated with matrigel for the invasion assay; (B) data are mean ± SE, n = 3; the number of cells per field were estimated.

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]. In particular, recent studies have shown that many extracts isolated from plants attenuate cancer metastasis and angiogenesis via down-regulating the expression of MMP-9 [16-18]. 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 non-stimulated 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 inhibitors with ubiquitin and subsequent phosphorylation/degradation [19]. In this study, we showed that MEPL inhibits TNF-α-induced DNA-binding.

CONCLUSION

The 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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COMPETING INTERESTS

The authors report no conflict of interest and they alone are responsible for the contents of this work.

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