Methanol extract of *Hydroclathrus clathratus* suppresses matrix metalloproteinase-9 in T24 bladder carcinoma cells by suppressing the NF-κB and MAPK pathways

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Received August 3, 2011; Accepted September 16, 2011

DOI: 10.3892/or.2011.1501

Abstract. *Hydroclathrus clathratus* is a brown marine seaweed and its extract possessing anti-cancer, anti-herpetic and anti-coagulant activities is a traditional drug and health food in Korea, Japan and China. However, little is known about the mechanism by which the methanol extract of *H. clathratus* (MEHC) inhibits invasion of cancer cells. In the present study, we investigated the effects of MEHC on the expression of matrix metalloproteinase-9 (MMP-9) in T24 human bladder carcinoma cells. Our findings showed that MMP-9 activity was significantly increased in response to tumor necrosis factor-α (TNF-α). However, treatment with MEHC substantially reversed TNF-α-induced MMP-9 activity. A matrigel invasion assay also showed that MEHC reduced TNF-α-induced invasion of T24 bladder carcinoma cells. We also found that MEHC significantly downregulated the expression of the *MMP-9* gene induced by TNF-α stimulation. Furthermore, we investigated the effects of MEHC on nuclear factor (NF)-κB activity, which is a potential transcriptional factor for regulating many invasive genes including MMP-9. MEHC suppressed NF-κB activity by suppressing IκB degradation and nuclear translocation of the NF-κB p65 and p50 subunits. TNF-α-induced phosphorylation of phosphatidyl-inositol 3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) was significantly downregulated in the presence of MEHC. Taken together, these results indicate that MEHC is a potential anti-invasive agent by suppressing TNF-α-induced cancer cell invasion and by specifically inhibiting NF-κB and MAPKs, as well as downstream target genes such as MMP-9.

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Key words: Hydroclathrus clathratus, matrix metalloproteinase-9, nuclear factor-κB, phosphatidyl-inositol 3 kinase/Akt, mitogen-activated protein kinase

Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the breakdown of the extracellular matrix under normal physiological conditions (1). According to recent studies, MMPs are also closely related to tumor invasion and metastasis by their capacity for tissue remodeling via the extracellular matrix as well as degradation of the basement membrane and induction of angiogenesis (1,2). MMPs are mainly secreted as zymogens and cleaved to their active forms, and their functions are tightly regulated by several different mechanisms (3). One of them, MMP-9 is a key effector that promotes tumor cell invasion through type-IV collagen degradation-dependent extracellular matrix remodeling (4). MMP-9 expression has been observed in tumors of various organs, including the bladder, brain, liver, prostate and pancreatic carcinoma (5). In particular, elevated MMP-9 expression in bladder tumor tissues is correlated with tumor stage, grade and prognosis (6). Complications in most bladder cancers are attributed to metastasis to distant organs including the regional lymph nodes, liver, lungs, bone, adrenal glands and intestines (7). Therefore, a good strategy to treat bladder cancers is to target MMP-9 inhibition.

A recent study has shown that the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways tightly regulate MMP-9 expression in several types of cancer cells (8). NF-κB is normally located in the cytoplasm as an inactivated dimer composed of p65 and p50 subunits (9). In response to pro-inflammatory stimuli, IκB is degraded by the phosphorylation and ubiqutination, and then NF-κB is released and translocated to the nucleus (10). Ultimately, NF-κB promotes the expression of tumor invasion-related genes such as MMP-9. The MAPKs are a group of signaling molecules that appear to play an important role in MMP expression. TNF-α stimulation is well known to result in the phosphorylation of p38, ERK and JNK leading to NF-κB activation in bladder cancer cells and induces tumor invasion (8). Therefore, the NF-κB and MAPK pathways are considered a good target to suppress MMP-9 expression to inhibit tumor invasion.

Hydroclathrus clathratus is a brown marine seaweed with anti-coagulant activity (11). The chemical constituents isolated

from this seaweed are commonly polysaccharides H3-a1 and H3-b1, which have anti-viral and anti-cancer properties (12). Some components of *H. clathratus* affect the growth of human acute promyelocytic leukemia, human breast carcinoma and human hepatocellular carcinoma cancer cell lines (13). In particular, phenolic compounds isolated various seaweeds predominantly possess anti-inflammatory and anti-proliferative activities through radical scavenging activity (14). Though the total polyphenol contents of seaweeds are interesting to assess anti-inflammatory and anti-proliferative activities, no reports have evaluated the effects of *H. clathratus* on MMP-9 gene expression in cancer cells.

In this study, we evaluated the effects of a methanol extract of *H. clathratus* (MEHC) on MMP-9 expression in TNF-α-stimulated T24 bladder carcinoma cells. We found that MEHC downregulated TNF-α-induced MMP-9 mRNA and protein expression by suppressing NF-κB activation and MAPK phosphorylation.

Materials and methods

Preparation of MEHC. MEHC was purchased from Jeju HI-Tech Industry Development Institute (Jeju, Republic of Korea). *H. clathratus* (stock no. AP060) was collected along the Jeju Island coast of Republic of Korea in July, 2006. Briefly, fresh *H. clathratus* was washed three times with tap water to remove salt, epiphyte and sand on the surface of the samples before storage -20˚C. 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). Polyclonal antibodies against MMP-9, p65, p50, IκBα, ERK, phospho (p)-ERK, JNK, p-JNK p38, p-p38, phosphatidyl-inositol 3 kinase (PI3K), p-PI3K, Akt and p-Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was obtained from Sigma. Roswell Park Memorial Institute medium (RPMI), antibiotics mixture and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Korea). Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul). Other chemicals were purchased as Sigma grades.

Total polyphenol contents. Total polyphenol contents of MEHC were quantified according to the methods of Yuan *et al* (15). Sample absorbaces were read at 720 nm. Gallic acid was used as the standard for a calibration curve. Total pholyphenol contents of the seaweed extracts were represented as gallic acid equivalents.

Cell culture and cell viability assay. T24 bladder carcinoma cells (ATCC, Rockville, MD, USA) were cultured at 37˚C in 5% CO_2 in RPMI supplemented with 10% FBS and antibiotics. In all experiments, cells were pretreated with MEHC 1 h before the addition of TNF-α (20 ng/ml) in serum-free RPMI. Cell viability was determined by an MTT assay. In brief, T24 bladder carcinoma cells ($1x10^5$ cells/ml) were plated onto 24-well plates and incubated overnight. The cells were treated with the indicated concentrations of MEHC for 1 h and then stimulated with TNF-α (20 ng/ml) for 24 h. Then, the cells were incubated with a solution of 0.5 mg/ml MTT and incubation for 45 min at 37˚C and 5% CO_2. 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 (iNtRON Biotechnology; Sungnam, Korea) according to the manufacturer's instruction. RNA (2 μg) was reverse-transcribed using MMLV reverse transcriptase (Bioneer; Daejeon, Korea). 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'-cca ccc atg gca aat tcc at-3' and reverse 5'-tct aga cgg cag gtc agg tcc acc-3'). Reaction products were analyzed on 1.0% agarose gels, and the bands were visualized by ethidium bromide.

Western blot analysis. Total cell extracts were prepared using PRO-PREP protein extraction kit (iNtRON Biotechnology). The preparation of cytoplasmic or nuclear extracts was conducted using NE-PER cytosolic/nuclear extraction reagents (Pierce; Rockford, IL, USA). 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 (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. The reaction mixture was electrophoretically separated on a 4% polyacrylamide gel in 0.5X Tris-borate buffer and transferred to a nylon membrane. The transferred DNA was 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.

Gelatin substrate gel zymography. T24 bladder carcinoma cells were incubated at 37˚C in 5% CO_2 in serum-free RPMI medium supplemented with 10% FBS and antibiotics with or without MEHC for 24 h. Supernatants were collected and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) copolymerized with gelatin. After electrophoresis, the gels were washed several times with 2.5% Triton X-100 for 1 h at room temperature to remove the 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 0.25% Coomassie blue 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. T24 bladder carcinoma cells were trypsinized and $5x10^4$ cells were placed onto matrigel-coated

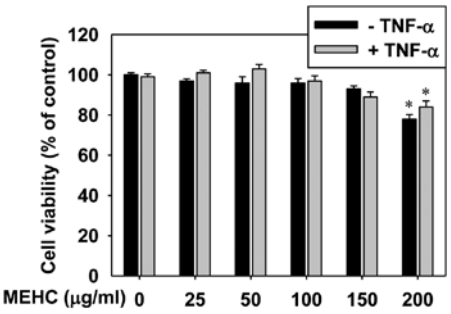

Figure 1. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on T24 cell viability. T24 bladder carcinoma cells ($1x10^5$ cells/ml) were incubated with the indicated concentrations of MEHC 1 h before tumor necrosis factor (TNF)-α (20 ng/ml) treatment for 24 h. Cell viability was determined by the MTT assay. Each value indicates the mean ± SE, and is representative of results obtained from three independent experiments. $^*p<0.05$ was considered significantly different from the values in cells with an untreated control.

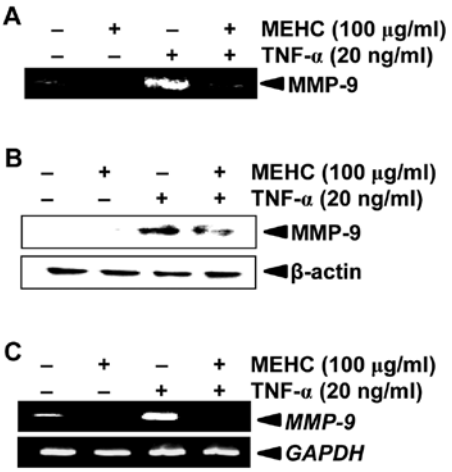

Figure 2. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on tumor necrosis factor (TNF)-α-induced matrix metalloproteinase-9 (MMP-9) expression in T24 bladder carcinoma cells. (A) T24 cells were treated with 100 μg/ml MEHC 1 h before TNF-α (20 ng/ml) treatment for 24 h. Conditioned 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. Cells were incubated with the indicated concentrations of MEHC 1 h before the TNF-α (20 ng/ml) treatment for 24 h. (C) Total RNA was isolated, and RT-PCR analysis of MMP-9 was performed. GAPDH and β-actin were used as internal controls for the RT-PCR and Western blot analysis, respectively. The experiment was repeated three times, and similar results were obtained.

transwell for 3 h. The cells were treated with 100 μg/ml of MEHC for 1 h and then stimulated with TNF-α (20 ng/ml) for 24 h. Then, the cells in the upper chamber were removed with a cotton swab. The cells that adhered on the outer surface of the transwell insert were fixed, stained with Coomassie blue, and counted under a light microscope.

Statistical analysis. All data were derived from at least three independent experiments. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat; Marine, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. 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. Statistical significance was regarded at $p<0.05$.

Results

Effects of MEHC on cell viability. As assayed by gallic acid equivalents, total phenol contents represented ~2.7±0.2% of MEHC. Next, in order to determine the effects of MEHC on cell viability in T24 bladder carcinoma cells, MTT assay was performed at 24 h after treatment with the indicated concentrations of MEHC in the presence or absence of TNF-α. MEHC alone at 25-150 μg/ml showed no cytotoxic effects (Fig. 1), but significant cytotoxicity was found at 200 μg/ml MEHC, regardless of the presence of TNF-α. DMSO (0.1%) as a solvent had no cytotoxic effect (data not shown). Therefore, the concentration of MEHC was applied within this range for further experiments.

Suppressive effect of MEHC on MMP-9 activity. Zymography, the real-time polymerase chain reaction (RT-PCR) and Western blot assays were conducted to assess whether MEHC regulates MMP-9 expression. The zymography data showed that MEHC significantly suppressed TNF-α-induced MMP-9 activity (Fig. 2A). We also found that MEHC significantly downregulated TNF-α-induced MMP-9 mRNA and protein expression. In the Western blot analysis, TNF-α stimulation of cells resulted in a significant increase in MMP-9 expression compared to that in the untreated control (Fig. 2B). However, MEHC reversed TNF-α-induced MMP-9 activity to the levels of the untreated control. Moreover, *MMP-9* gene expression was confirmed by RT-PCR analysis. Pretreatment with MEHC significantly suppressed TNF-α-induced *MMP-9* upregulation at the mRNA level (Fig. 2C). Taken together, these results indicate that MEHC suppressed upregulation of TNF-α-stimulated MMP-9 expression at the transcriptional level.

Inhibitory effect of MEHC on invasion of T24 bladder carcinoma cells. MMP-9 is thought to be critically involved in the processes of tumor invasion and angiogenesis (5). Because MEHC inhibited MMP-9 expression and activity, we examined the effects of MEHC on invasion of T24 bladder carcinoma cells (Fig. 3A). When the cells were treated with TNF-α alone, a remarkable 3-fold higher increase in cell invasion was observed compared to that in the untreated control. However, MEHC pretreatment resulted in an ~50% reduction in penetration through a matrigel-coated membrane compared to that in the TNF-α-treated group (Fig. 3B). These results confirm that MEHC inhibited TNF-α-induced invasion in T24 bladder carcinoma cells.

Inhibitory effects of MEHC on TNF-α-induced NF-κB activity. We assessed the specific DNA-binding activity of NF-κB using an electrophoretic mobility shift assay (EMSA) to investigate whether MEHC inhibits MMP-9 activity by suppressing NF-κB activity. TNF-α stimulation caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA at 30 min; however, pretreatment with MEHC for 1 h significantly reduced TNF-α-induced NF-κB

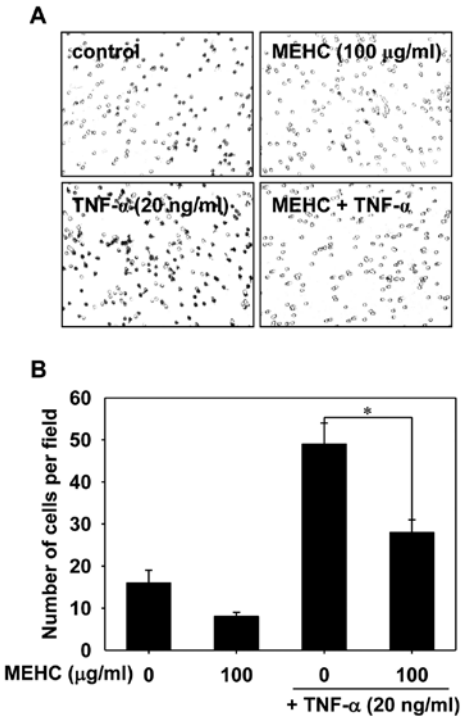

Figure 3. Suppressive effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on invasion of T24 bladder carcinoma cells. (A) The upper parts of the transwells were coated with matrigel for the invasion assay. Then, the cells were cultured in serum-free media for 1 h before treatment with MEHC (100 μg/ml) in the absence or presence of tumor necrosis factor (TNF)-α (20 ng/ml). After 24 h, the numbers of cells passing through the matrigel to the membrane were dyed using 0.125% Coomassie blue in ethanol. (B) Data are expressed as overall mean ± SE from three independent experiments, and the numbers of cells per field are estimated. Statistical significance was determined by two-way ANOVA test ($^*p<0.05$ vs. TNF-α-treated group).

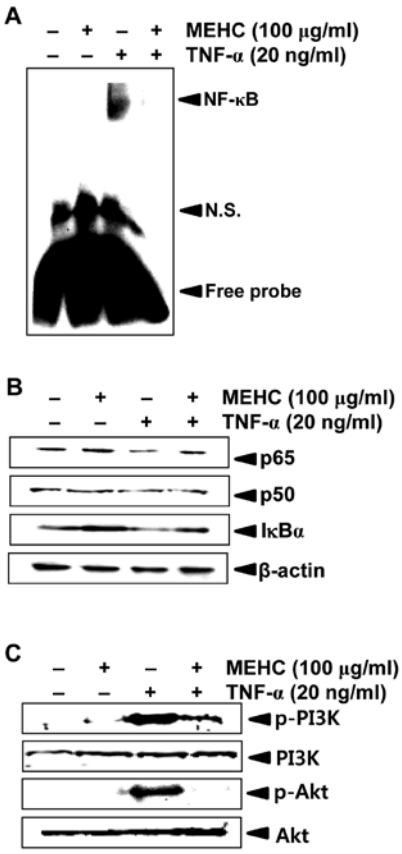

Figure 4. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on nuclear factor (NF)-κB DNA binding activity. Cells were preincubated with MEHC (100 μg/ml) 1 h before TNF-α (20 ng/ml) stimulation for 30 min. (A) Nuclear extracts were assayed for NF-κB activity using an electrophoretic mobility shift assay and (B) the levels of p50, IκB and p65 were analyzed by Western blot analysis. (C) In a parallel experiment, protein lysates were prepared, subjected to SDS-PAGE and immunoblotted using antibodies against PI3K, Akt and their phosphorylated forms. β-actin was used as an internal control for Western blot assays. The experiment was repeated three times, and similar results were obtained. N.S., non-specific.

activity (Fig. 4A). In a parallel experiment, p65, p50 and IκBα expression levels in the cytoplasmic region were determined after TNF-α stimulation in the absence or presence of MEHC (Fig. 4B). TNF-α significantly decreased p65, p50 and IκBα expression in the cytoplasmic compartment of T24 bladder carcinoma cells. However, treatment with MEHC sustained the TNF-α-induced expression of p65, p50 and IκBα (Fig. 4B). Because the PI3K and Akt signal pathways regulate NF-κB activity upstream, we evaluated whether MEHC regulates TNF-α-induced phosphorylation of this signaling pathway. As expected, stimulation with TNF-α significantly induced PI3K and Akt phosphorylation; however, pretreatment with MEHC shifted the expression level of the untreated control (Fig. 4C). These results indicate that MEHC inhibited the suppression of NF-κB activity by suppressing the PI3K/Akt signal pathway.

Inhibitory effects of MEHC on TNF-α-induced MAPK phosphorylation. We next evaluated the effects of MEHC on TNF-α-induced phosphorylation of ERK, p38 and JNK in T24 bladder carcinoma cells. The phosphorylation levels of the various MAPKs increased dramatically 30 min after stimulation with TNF-α. MEHC significantly reduced TNF-α-induced phosphorylation of ERK, p38 and JNK. However, non-phosphorylated ERK, p38 and JNK expression was unaffected by TNF-α and/or MEHC alone. These results indicate

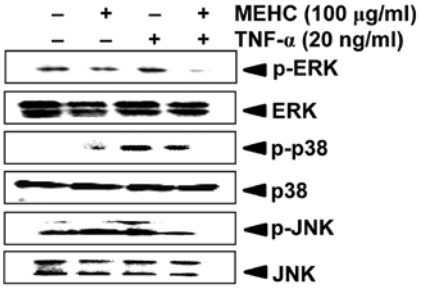

Figure 5. Effects of a methanol extract of *Hydroclathrus clathratus* (MEHC) on mitogen-activated protein kinases (MAPKs) in TNF-α-stimulated T24 bladder carcinoma cells. T24 cells were pretreated with 100 μg/ml MEHC for 1 h and then incubated with TNF-α (20 ng/ml) for 30 min. Total cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies against ERK, JNK, p38 and their phosphorylated forms. The experiment was repeated three times, and similar results were obtained. Statistical significance was determined by two-way ANOVA test ($^*p<0.05$ vs. TNF-α-treated group).

that MEHC may be involved in inhibiting MAPK activity to suppress TNF-α-induced invasion (Fig. 5).

Discussion

Our previous study suggested that MEHC considerably suppresses inflammatory responses such as lipopolysaccharide-induced pro-inflammatory cytokines and nitric oxide expression in BV2 microglia cells (unpublished data). It has also been reported that MEHC inhibits the proliferation of a variety of human cancer cells, including human breast carcinoma (MCF-7) and human hepatocellular carcinoma (13). Nevertheless, it is not fully known how MEHC regulates anti-cancer activity during the invasion process. Therefore, we determined that MEHC suppressed TNF-α-induced NF-κB activity and this downregulation lead to decreased invasion of bladder cancer cells through the regulation of MMP-9. This study provides substantial evidence that MEHC contained common levels of phenol content compared to other seaweed (~3% in extracts) and inhibited TNF-α-induced MMP-9 expression in T24 bladder carcinoma cells by suppressing NF-κB activity through the PI3K/Akt and MAPK pathways.

Tumor metastasis is a multistep process by which a subset of individual cancer cells disseminates from a primary tumor to distant secondary organs or tissues. MMPs play a major role promoting tumor metastasis (16,17). One of them, MMP-9, is regarded as a critical molecule to eliminate the progression of tumor invasion. Thus, the inhibitory effect of MMP-9 expression is important as a therapeutic experimental model of tumor invasion. Our results demonstrated that MEHC inhibited TNF-α-induced MMP-9 activity accompanied by the suppression of MMP-9 gene transcription in T24 bladder carcinoma cells. Interestingly, the matrigel assay showed that MEHC suppressed cell invasion. MMP-9 is synthesized and secreted into the extracellular matrix as a late pro-enzyme, and the pro-peptide domain, containing a cysteine switch, interacts with zinc at the active site for enzyme activation by regulatory proteins such as tissue inhibitor of MMP (TIMP) and MT1-MMP (18-20). Therefore, whether MEHC-induced MMP-9 downregulation is regulated by TIMPs and MT1-MMP in the extracellular matrix needs to be investigated.

Several studies have identified the signal transduction pathways that are involved in regulating MMP-9 expression in tumor cells (21,22). In particular, NF-κB is an important transcription factor for regulating the *MMP-9* gene promoter and contains NF-κB binding sites (23). Once NF-κB is fully activated, it participates in the regulation of various target genes in different cells and is involved in various functions (9,24). NF-κB is a heterodimer consisting of p65 and p50 proteins; it is located in the cytosol and is complexed with the IκBα inhibitory protein (25). A variety of extracellular signals including TNF-α and growth factors activate IκB kinase, which results in ubiquitination and degradation of IκBα by proteasomes and eventual dissociation of IκBα from NF-κB (10). The activated NF-κB is then translocated to the nucleus where it binds to specific DNA sequences. Ultimately, NF-κB promotes the expression of various genes including MMP-9 (26). In this study, we showed that MEHC inhibited p65 and p50 protein translocation by suppressing IκBα degradation. However, the human MMP-9 promoter also contains two other transcription factors such as AP-1 and Sp1 (27,28). In particular, AP-1 binding to the MMP-9 promoter region is thought to be important for regulating MMP-9 expression in response to phorbol 12-myristate 13-acetate (PMA); however, many researchers have reported that AP-1 activation is not involved in TNF-α-stimulated MMP-9 expression (29,30). MMP-9 expression is also regulated by Sp1 in response to PMA and TNF-α (28,31). Therefore, further study is required to determine which transcriptional factors are inhibited in MEHC-induced MMP-9 downregulation.

We investigated whether MEHC inhibits PI3K and Akt phosphorylation, because PI3K and Akt are upstream regulators of NF-κB (32,33). Treatment with MEHC suppressed PI3K and Akt phosphorylation. These data indirectly support the result that the TNF-α-induced PI3K/Akt/NF-κB/MMP-9 pathway was inhibited by treatment with MEHC. In a previous study, Zhu *et al* reported that these signaling pathways are highly activated during the progression of chronic myeloid leukemia (34). Additionally, the role of MAPKs in the regulation of MMP-9 expression is particularly well understood in TNF-α-stimulated cancer cells (35,36). Some researchers have reported that TNF-α-induced p38 is a MAPK effector that induces MMP-9 expression regardless of ERK1/2 and JNK (35,36). In contrast, other have shown that TNF-α induces MMP-9 expression through AP-1-dependent ERK1/2 and/or JNK activation (36). In this study, we found that MEHC suppressed TNF-α-induced ERK, p38 and JNK phosphorylation, whereas non-phosphorylated ERK, p38 and JNK kinase expression was unaffected by MEHC treatment. Presumably, this discrepancy with previous studies was due to the cell types and specificity of the chemicals. In conclusion, our results showed that MEHC is a potent inhibitor of TNF-α-induced MMP-9 expression and invasion by suppressing the PI3K/Akt/NF-κB pathway.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (no.: 2011-0001048).

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