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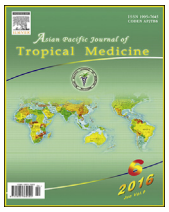

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Methanol extract of *Codium fragile* inhibits tumor necrosis factor-α-induced matrix metalloproteinase-9 and invasiveness of MDA-MB-231 cells by suppressing nuclear factor-κB activation

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ABSTRACT

Objective: To evaluate whether the methanol extract of *Codium fragile* (MECF) regulates tumor necrosis factor-α (TNF-α)-induced invasion of human breast cancer MDA-MB-231 cells by suppressing matrix metalloproteinase-9 (MMP-9).

Methods: Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis were performed to analyze the expression of MMP-9 and nuclear factor-κB (NF-κB) subunits, p65 and p50, and IκB in MDA-MB-231 cells. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used for cell viability. MMP-9 activity and invasion were measured by gelatin zymography and a matrigel invasion assay, respectively. NF-κB activity was measured by an electrophoretic mobility shift assay and luciferase activity.

Results: MECF had no effect on cell viability up to a concentration of 100 μg/mL in human breast cancer MDA-MB-231 cells regardless of the presence of TNF-α. MDA-MB-231 cells that were stimulated with TNF-α showed a marked increase of invasion compared to the untreated control, whereas pretreatment with MECF downregulated the TNF-α-induced invasion of MDA-MB-231 cells. Additionally, zymography, western blot analysis, and RT-PCR confirmed that MECF decreased TNF-α-induced MMP-9 expression and activity which is a key regulator for cancer invasion. According to an electrophoretic morbidity shift assay, pretreatment with MECF in MDA-MB-231 cells significantly decreased the TNF-α-induced DNA-binding activity of NF-κB, which is an important transcription factor for regulating cancer invasion-related genes such as MMP-9. Furthermore, treatment with MECF sustained the expression of p65 and p50 in response to TNF-α in the cytosolic compartment. The luciferase assay demonstrated that MECF attenuated TNF-α-induced NF-κB luciferase activity.

Conclusion: MECF exhibited its anti-invasive capability by downregulating TNF-α-induced MMP-9 expression, resulting from the suppression of NF-κB activity in the human breast cancer cell line MDA-MB-231.

1. Introduction

Metastasis and invasion are key factors that determine the malignant behavior of cancer cells. In particular, extracellular proteinases are essential for the metastasis and invasion processes that degrade the components of the extracellular matrix (ECM) and facilitate the disconnection of intercellular adhesions and separation of single cells from solid tumor tissue to

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metastatic colonies at distant sites [1]. Matrix metalloproteinases (MMPs) are important zinc-dependent endopeptidases responsible for the degradation of major components of ECM, including type IV and V gelatin, and collagen. Thus, its role is closely related to the metastasis and invasion of cancer cells [2]. Among the MMP family, which consists of 24 members, MMP-9 is known to be significantly upregulated in almost all tumor types [3]. Furthermore, MMP-9 expression positively correlates with cancer stage, grade, and prognosis [4]. Several studies have shown that cancer cells that were attributable to metastasis to distant organs, including the lungs, liver, lymph nodes, and adrenal medulla expressed high levels of MMP-9 [3,5]. This suggests that MMP-9 is not only important in identifying invasion symptoms and its diagnosis, but also a promising strategy to prevent cancer invasion and metastasis. Therefore, MMP-9 expression may prove an important therapeutic target for preventing the invasion and metastasis of a broad-spectrum of cancers.

Tumor necrosis factor alpha (TNF-α) plays a key role in tumorigenesis by enhancing the growth of cancer and its survival, invasion, and metastasis, resulting in a direct network of MMP-9 production [6,7]. After TNF-α binds to its receptor, adapter molecules are recruited to the conformationally-changed receptor, allowing the initiation of the intracellular signaling pathways including nuclear factor-κB (NF-κB) [8]. In particular, NF-κB is not only well known to contribute to cancer invasion and resistance to chemotherapy, but also to be constitutively activated in various cancer [9,10]. Until cells receive an appropriate stimulus, NF-κB complexes are sequestered by binding with the inhibitor kappa B (IκB) in the cytoplasm, forming a transcriptionally inactive stage [11]. In response to various stimuli such as TNF-α, IκB is phosphorylated and degraded, resulting in the liberation of NF-κB, which allows NF-κB to accumulate in the nucleus and thus activates the expression of target genes such as MMP-9 [12,13]. Therefore, the inhibition of NF-κB can be considered as an adjuvant approach to suppress MMP-9 expression and thus prevent cancer invasion and metastasis.

Marine macroalgae are an inexhaustible source of unique natural products with pharmacological activities, which produce a vast array of bioactive secondary metabolites [14]. The secondary metabolites from marine macroalgae were recently demonstrated to possess anti-tumor and anti-inflammatory capabilities [15]. *Codium fragile* (*C. fragile*) is a green macroalgae belonging to family Codiaceae, which has demonstrated anti-cancer activity against various cancers. Previously, Noda *et al*, reported that the *C. fragile* extract exhibited a 43.4% cancer growth inhibition rate in mice [16]. Furthermore, clerosterol from *C. fragile* enhanced cytotoxicity in human melanoma cells [17]. Additionally, aqueous extracts of *C. fragile* exhibited strong cytotoxic activities against the CT26 mouse colon carcinoma cell line and carotenoids from *C. fragile* induced apoptosis in human leukemia cells [18,19]. However, no study has investigated the effect of *C. fragile* on MMP-9 expression and activity or invasion and metastasis in TNF-α- stimulated cancer cells.

In the present study, we examined effects of the methanol extract of *C. fragile* (MECF) on MMP-9 expression and activity in TNF-α-stimulated MDA-MB-231 human breast cancer cells. Our results indicate that MECF suppressed TNF-α-induced MMP-9 expression and activity by downregulating the NF-κB pathway, leading to the inhibition of MDA-MB-231 invasion.

2. Materials and methods

2.1. Preparation of C. fragile

MECF was purchased from Jeju Hi-Tech Industry Development Institute (Jeju, Republic of Korea). In brief, *C. fragile* was collected along the Jeju Island coast (Republic of Korea). Fresh *C. fragile* was washed three times with tap water to remove salt, epiphyte, and sand on the surface of the samples before storage at −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 the vacuum. The extract was filtered through 0.45 μm filter and the filtrate was freeze-dried (yield, approximately 4.1 g) and stored at 4 °C. The dried filtered through 0.22 μm filter before use.

2.2. Antibodies and reagents

Antibodies against p50, p65, IκB, β-actin, and nucleolin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against MMP-9 was obtained from Cell Signaling (Beverly, MA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin, and TNF-α were purchased from KOMA Biotechnology (Seoul, Republic of Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO). RPMI 1640 medium, fetal bovine serum (FBS), and antibiotics mixture were purchased from WelGENE (Daegu, Republic of Korea).

2.3. Cell culture and viability

Human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured at 37 °C in a 5% CO_2-humidified incubator and maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% antibiotics mixture. The cells were seeded at 5×10^4 cells/ml, incubated, and then treated with the indicated concentrations of MECF in the presence or absence of TNF-α (20 ng/mL). Following 24-h incubation, the viability was measured by MTT assay.

2.4. Invasion assay

Invasion assay was performed into 24-well chambers (Corning, Corning, NY) coated with matrigel in serum-free RPMI 1640 medium. Precoated filters (6.5 mm in diameter and 8 μm pore size) were rehydrated and the cells (5×10^4 cells/mL) in medium with or without MECF in the presence of TNF-α (20 ng/mL) were seeded into the upper part of each chamber. Following 24-h incubation, non-migrated cells were removed using cotton swabs and migrated cells were fixed and stained with 0.125% Coomassie Blue in a methanol:acetic acid: distilled water mixture (15:10:45, v/v/v). Invading cells were counted under a light microscope.

2.5. Gelatin zymography assay

Cultured MDA-MB-231 cells were collected and washed three times with serum-free RPMI 1640 medium. The cells were seed at 3×10^5 cells/mL in serum-free RPMI 1640

medium and treated with MECF in the presence of TNF-α (20 ng/mL). The medium was collected and electrophoresed on a polyacrylamide gel containing 0.1% (w/v) gelatin as a substrate. The gel was washed at room temperature for 10 min, 3 times with 2.5% Triton X-100 washing buffer, and subsequently incubated at 37 °C for 24 h in reaction buffer (0.2% Brij, 35.5 mM $CaCl_2$, 1 mM NaCl, and 50 mM Tris, pH 7.4). The gel was stained for 1 h with 0.25% Coomassie Blue in 10% acetic acid/40% methanol and then destained with 10% acetic acid/40% methanol for 1 h. Areas of protease activity appeared as clear bands.

2.6. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Easy-Blue reagent (iNtRON Biotechnology, Sungnam, Korea). RNA concentration and purity were measured by absorbance at 260 nm/280 nm. Genes of interest were amplified from cDNA that was synthesized from 1 μg total RNA using MMLV reverse transcriptase (Bioneer Corp., Daejeon, Korea). Primers for glyceraldenhyde-3-phosphate dehydrogenase (GAPDH) (sense 5′-CCA CCC ATG GCA AAT TCC ATG CA-3′ and antisense 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′) and MMP-9 (sense 5′-CCT GGA GAC CTG AGA ACC AAT CT-3′ and antisense 5′-CCA CCC GAG TGT AAC CAT AGC-3′) were used. PCR was performed at 94 °C for 5 min followed 27 cycles of 94 °C for 30 s, annealing temperature (*GAPDH* at 62 °C and *MMP-9* at 59 °C) for 30 s, 72 °C for 30 s followed via final extension at 72 °C for 5 min. Following amplification, PCR products were separated 1% agarose gels and visualized using ethidium bromide fluorescence.

2.7. Western blot analysis

Total protein extracts were prepared by a PRO-PREP protein extraction kit (iNtRON Biotechnology). Briefly, MDA-MB-231 cells were harvested, washed twice with ice-cold PBS, and gently lysed for 30 min in 100 μL ice-cold PRO-PREP lysis buffer. The lysates were centrifuged at

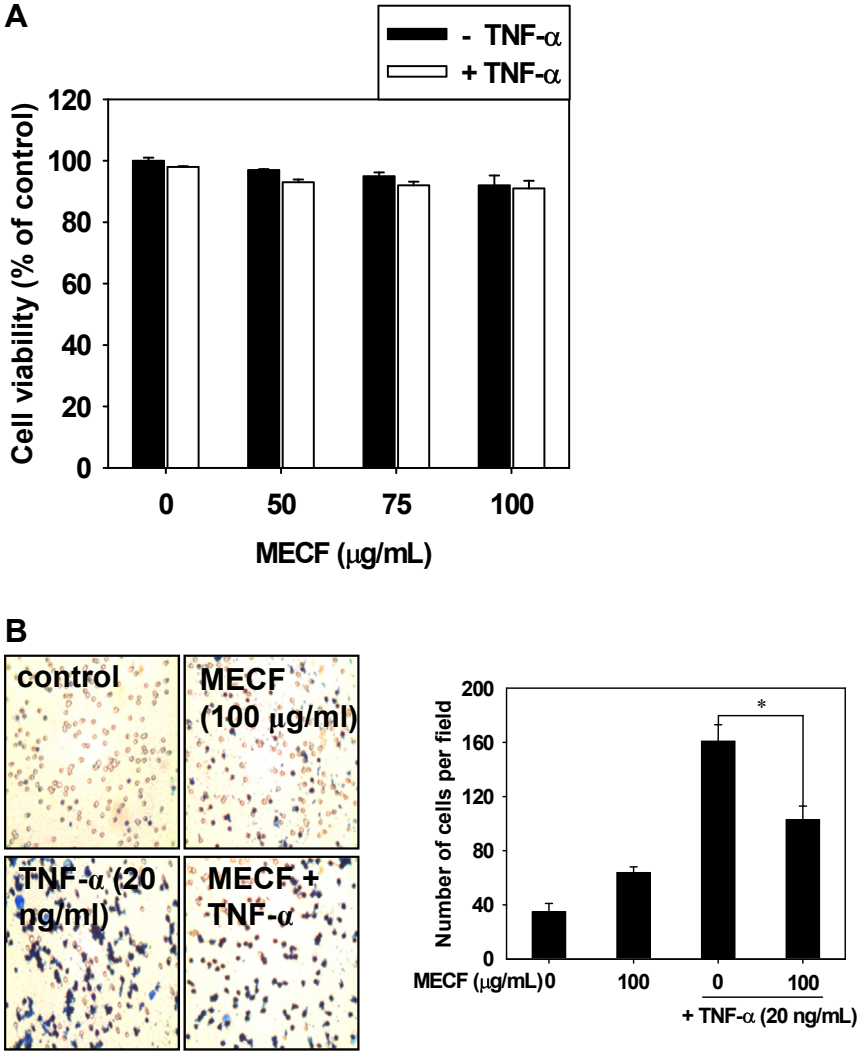

Figure 1. Effects of MECF on the viability and invasion activity of MDA-MB-231 cells.
(A) MDA-MB-231 cells (5×10^4 cells/mL) were cultured in 24-well plates and treated with the indicated concentrations (0–100 μg/mL) of MECF in the presence or absence 20 ng/mL TNF-α for 24 h. Cell viability was determined by an MTT assay. (B) For the invasion assay, the upper compartments of transwells were coated with matrigel. MDA-MB-231 cells were cultured in serum-free media for 1 h before treatment with MECF (100 μg/mL) in the presence or absence of TNF-α (20 ng/mL) for 24 h. The cells that passed through the matrigel to the membrane were stained using 0.125% Coomassie blue in ethanol. *$P < 0.05$ *vs*. TNF-毯-treated group.

14 000 *g* at 4 °C for 10 min. Supernatants were collected and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The samples were stored at −80 °C or immediately used for Western blot analysis. The proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Proteins were detected by an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

2.8. Electrophoretic mobility shift assay (EMSA)

The cytoplasmic and nuclear proteins were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). DNA-binding activity of NF-κB was performed using nuclear extracts. Synthetic complementary NF-κB-binding oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Santa Cruz Biotechnology) were biotinylated using biotin 3′-end DNA labeling kit (Pierce) and annealed for 30 min at 37 °C according to the manufacturer's instructions. Labeled oligonucleotides, 10 μg nuclear protein extracts, 100 ng poly[deoxyinosinic-deoxycytidylic] acid, and LightShiftTM chemiluminescent EMSA kit mixture were then incubated for 30 min at room temperature. The samples were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 1 h in 0.5 × Tris-borate/EDTA (TBE) buffer and transferred onto a positively charged nylon membrane (HybondTM-N^+) in 0.5 × TBE buffer at 100 V for 1 h on ice. Transferred DNAs were cross-linked on the membrane at 120 mJ/cm^2 and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer's instructions.

2.9. Luciferase activity assay

NF-κB reporter construct was purchased from Clontech (Palo Alto, CA). In brief, MDA-MB-231 cells were seeded onto 6-well plates at a density of 3 × 10^5 cells/mL and incubated overnight. The cells were transfected with NF-κB reporter construct by Lipofectamine method. After 6-h incubation, the cells were washed and cultured in RPMI 1640 containing 10% FBS with MECF (100 μg/mL) in the presence or absence of TNF-α (20 ng/mL). The cells were lysed with lysis buffer (2 mM DTT, 150 mM NaCl, 20 mM Tris–HCl, and 1% Triton X-100) at 24 h. The lysate was mixed with luciferase activity reagent, and luminescence was measured using a GLOMAX luminometer (Promega, Madison, WI) and intensity of luminescence was obtained using an image analyzer.

2.10. Statistical analysis

The images were visualized with Chemi-Smart 2000 (VilberLourmat, Marine, Cedex, France). Images were captured using Chemi-Capt (VilberLourmat) and transported into Photoshop. All bands were shown a representative obtained in three independent experiments and quantified by Scion Imaging software (http://www.scioncorp.com). Statistical analyses were conducted using SigmaPlot software (version 12.0). Values were presented as mean ± standard error (S.E.). Significant differences between the groups were determined using the unpaired one-way ANOVA test by Bonferroni's test. Statistical significance was regarded at $P < 0.05$.

3. Results

3.1. MECF inhibits TNF-α-induced cell invasion

In order to measure whether MECF influences cell viability, we treated MDA-MB-231 cells with various concentrations of MECF in the presence or absence of TNF-α for 24 h and then performed MTT assay. Exposure up to 100 μg/mL MECF had no effect on cell viability in the presence or absence of TNF-α (Figure 1A). Furthermore, to examine the inhibitory effects of MECF on the invasion of MDA-MB-231 cells, we performed an invasion assay using matrigel-coated transwells. TNF-α-stimulated MDA-MB-231 cells showed a marked increase of invasion compared to that of the untreated control; however, pretreatment with MECF downregulated the TNF-α-induced invasion of the cells (Figure 1B). Therefore, these results indicate that MECF inhibited TNF-α-induced invasion in MDA-MB-231 cells without any direct cytotoxicity.

3.2. MECF downregulates TNF-α-induced MMP-9 activity and expression

MMP-9 is a key enzyme for the invasion and metastasis of cancer cells. Subsequently, in order to evaluate whether MECF

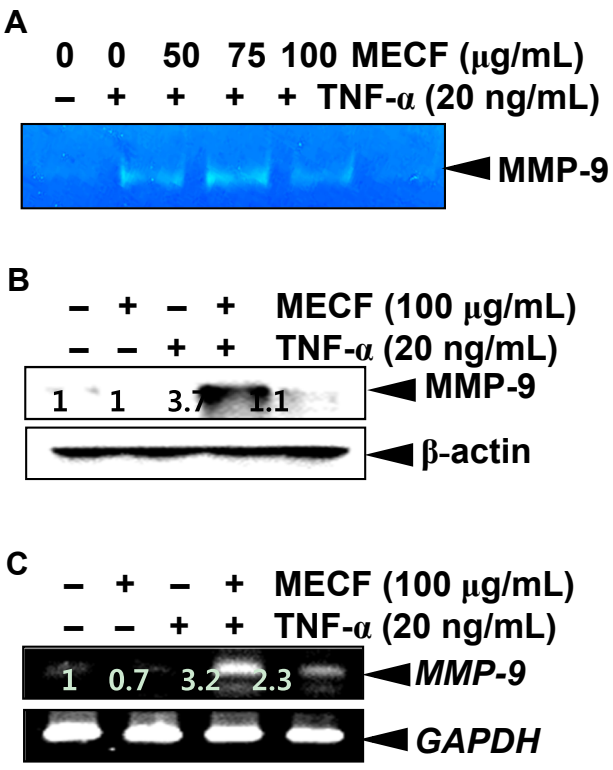

Figure 2. Effects of MECF on TNF-α induced MMP-9 expression and activity in MDA-MB-231 cells.
(A) MDA-MB-231 cells were treated with the indicated concentrations (0–100 μg/mL) of MECF for 1 h before treatment with 20 ng/mL TNF-α in the absence of FBS for 24 h. The conditioned medium was collected from cultures and subjected to 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) MDA-MB-231 cells were incubated with the indicated concentrations (0–100 μg/mL) of MECF for 1 h before treatment with TNF-α (20 ng/mL) for 6 h. Total RNA was isolated, and RT-PCR analysis of MMP-9 was performed. GAPDH and β-actin were used as internal controls for RT-PCR and Western blot analysis, respectively.

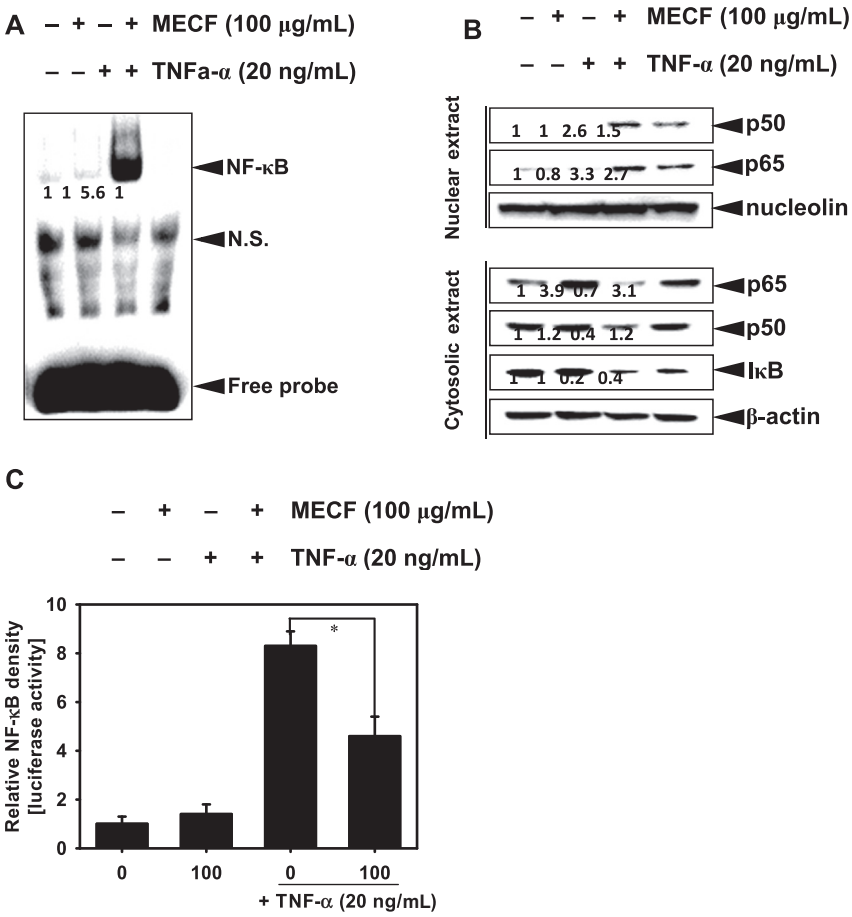

Figure 3. Effect of MECF on TNF-α-induced NF-κB activity in MDA-MB-231 cells. MDA-MB-231 cells were treated with MECF (100 μg/mL) in the presence of TNF-α (20 ng/mL) and nuclear and cytosolic extracts were extracted.
(A) The nuclear extracts were prepared after 30 min, and NF-κB binding to its DNA promoter region in the extract was measured using an EMSA. (B) Levels of p65, p50, and IκB in the nuclear (top) and cytosolic (bottom) extracts were determined by Western blot analysis at 30 min β-Actin and nucleolin were used as cytosolic and nuclear internal controls for the Western blot analysis. (C) MDA-MB-231 cells were transfected with the NF-κB promoter-containing reporter vector and treated with MECF (100 μg/mL) in the presence of TNF-α (20 ng/mL). Luciferase activity was measured by a luminometer. (*$P < 0.05$ *vs*. TNF-α-treated group).

regulates MMP-9 activity and expression, we performed zymography, Western blot analysis, and RT-PCR. According to the zymography data, TNF-α-treated MDA-MB-231 cells significantly increased the gelatin-degrading activity of MMP-9 whereas MECF-pretreated cells decreased the TNF-α-induced MMP-9 activity in a concentration-dependent manner (Figure 2A). In addition, the Western blot analysis revealed that stimulation with TNF-α resulted in a remarkable upregulation of MMP-9 expression in the protein level compared to that of the untreated control; however, pretreatment with MECF inhibited TNF-α-induced MMP-9 expression (Figure 2B). In accordance with the suppression of MMP-9 activity and protein expression of MECF, RT-PCR analysis also showed that MECF significantly downregulated TNF-α-induced *MMP-9* expression in MDA-MB-231 cells (Figure 2C). These data suggests that MECF inhibited MMP-9 expression and activity, leading to the suppression of invasion of MDA-MB-231 cells.

3.3. MECF suppresses TNF-α-induced NF-κB

To further examine whether MECF suppresses MMP-9 expression by inhibiting NF-κB, we determined the specific DNA-binding activity of NF-κB. According to EMSA data, the TNF-α-induced DNA-binding activity of NF-κB at 30 min was 5-fold to that of the untreated control; however, pretreatment with MECF significantly decreased TNF-α-induced NF-κB activity (Figure 3A). Consistent with EMSA data, Western blot analysis also showed that treatment with TNF-α increased p65 and p50 levels in the nuclear compartment, whereas MECF attenuated p65 and p50 levels in the nuclear extract (Figure 3B). In a parallel experiment, TNF-α decreased p65, p50, and IκB expression in the cytosol which suggests that TNF-α induces nuclear translocation of p65 and p50 by degrading IκB. However, treatment with MECF sustained p65, p50, and IκB in response to TNF-α in the cytosolic compartment. Moreover, we examined NF-κB promoter activity using transiently transfected MDA-MB-231 cells with a luciferase reporter vector consisting of NF-κB binding sites. The TNF-α-stimulated MDA-MB-231 cells showed approximately a 7-fold increases in NF-κB luciferase activity when compared to the untreated control (Figure 3C). Interestingly, pretreatment with MECF decreased TNF-α-induced NF-κB luciferase activity. Taken together, these results indicated that MECF attenuated TNF-α-induced NF-κB activity, resulting in MMP-9 gene expression.

4. Discussion

Recent studies reported that the *C. fragile* extract exhibits diverse pharmacological properties such as anti-edema, anti-viral, anti-bacterial, anti-allergic, anti-protozoal, and anti-

carcinogenic activity [20,21]. Nevertheless, there are no reports on the anti-invasive properties of the *C. fragile* extract. Therefore, we investigated the anti-invasive activity of MECF in MDA-MB-231 cells. The present study showed that MECF suppressed TNF-α-induced MMP-9 expression and activity by inhibiting NF-κB activity, resulting in the anti-invasion of MDA-MB-231 cells.

Numerous reports have demonstrated that various MMPs are directly implicated in the cancer cell invasion process and therefore promote metastasis [1,3]. In particular, MMP-9 is implicated in carcinogenesis as a key enzyme that is overexpressed in different types of malignant cancers, and its expression and activity are associated with the aggressiveness of cancer cells [5]. Many studies also reported that elevated levels of MMP-9 are found in colorectal, prostate, brain, lung, breast, and ovarian cancers [22–24]. MMP-9 is directly involved in the degradation of the collagen IV component of the basement membrane, which plays a crucial role in cancer invasiveness [3]. According to our results, MECF suppressed TNF-α-induced MMP-9 expression and activity in MDA-MB-231 cells. Furthermore, the matrigel assay showed that MECF markedly downregulated TNF-α-induced invasion of the MDA-MB-231 cells, which suggests that MECF is a promising strategy for regulating cancer invasion. Additionally, the activity of MMP-9 is traditionally postulated to be governed by tissue inhibitors of metalloproteinases (TIMPs), which are complex proteins composed of four proteins ranging in molecular size from 21 to 28 kDa [25]. Therefore, it is essential to understand whether TIMPs may be involved in MECF-induced MMP-9 regulation.

In past decades, numerous *in vivo* and *in vitro* studies indicated that the pleiotropic NF-κB plays a significant role in various types of biological activities including carcinogenesis, adhesion, invasion, and cell survival [26]. In particular, NF-κB regulates the expression of inflammatory and carcinogenic genes including MMP-9 in different types of cancers such as breast, lung, colon, hepatocellular, prostate and ovarian carcinomas [24]. Therefore, targeting NF-κB activity is a possible method to attenuate cancer invasion by suppressing MMP-9 expression. Accordingly, recent studies have focused on macroalgae- and plants-derived natural compounds that suppress MMP-9 expression via the NF-κB signaling pathway [15,27]. The results from this present study demonstrated that MECF inhibited the invasiveness of MDA-MB-231 cells by downregulating MMP-9, which resulted from the suppression of NF-κB activity.

In conclusion, we investigated the anti-invasive property of MECF in MDA-MB-231 cells, through its suppression of NF-κB-dependent MMP-9 activity. Our results indicate that MECF might be a promising therapeutic candidate for the inhibition of cancer invasiveness through the suppression of NF-κB and the downstream target, MMP-9.

Conflict of interest statement

The authors declare to have no conflict of interest at all.

Acknowledgment

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