

Cordycepin inhibits TPA-induced matrix metalloproteinase-9 expression by suppressing the MAPK/AP-1 pathway in MCF-7 human breast cancer cells

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Received September 28, 2009; Accepted November 24, 2009

DOI: 10.3892/ijmm_00000338

Abstract. Matrix metalloproteinase-9 (MMP-9), which degrades the extracellular matrix (ECM), plays an important role in breast cancer cell invasion. NF- κ B and AP-1 are known to induce MMP-9 expression. We investigated whether cordycepin, an NF- κ B or AP-1 inhibitor, can modulate MMP-9 expression and cell invasion in MCF-7 cells. Toxicity of cordycepin was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MMP-9 expression was determined by real-time PCR, Zymography, and Western blot analysis. AP-1 activation was assayed by electrophoretic mobility shift assay (EMSA). MAPK signaling was evaluated by Western blotting with specific p-ERK, and ERK, p-p38, p38, p-JNK, JNK antibodies. Cordycepin suppressed AP-1 activation, but not NF- κ B activation in 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated MCF-7 cells. Cordycepin inhibits TPA-induced MMP-9 expression and cell invasion by suppressing AP-1 activation. Also, cordycepin suppressed the MAPK signaling pathway. Cordycepin is a potent inhibitor of TPA-induced MMP-9 expression and blocks strongly the ability of AP-1 activation via MAPK signaling pathway in MCF-7 cells.

Introduction

The Cordyceps mushroom, *Cordyceps sinensis*, is the most explored species followed by *Cordyceps militaris* (1-3). Recent studies have shown that extract of *Cordyceps* exhibits anti-tumor effects on cancers of the bladder, colon, lung, and fibrosarcoma (4), as well as inhibitory effects on the production of inflammatory mediators (5). *Cordyceps* and products are available in Western countries as over-the-counter medicine/tonics that advertise them as Chinese herbs with anti-aging, 'pro-sexual', anti-cancer and immune-boosting effects (3). Cordycepin, which is a 3-deoxyadenosin isolated from *Cordyceps*, has been reported to exert inhibitory effects on macrophages based on anti-inflammatory properties (6). However, the anti-metastatic effects of cordycepin have not yet been reported.

Invasion and metastasis are the fundamental properties and major causes of morbidity and mortality in breast cancer patients. These processes require degradation of the extracellular matrix (ECM), which provides biochemical and mechanical barriers to cell movement in cancer cells (7). ECM consists of type IV collagen, laminin, heparan sulfate proteoglycan, nidogen and fibronectin (8). ECM degradation requires extracellular proteinases, of which the matrix metalloproteinases (MMPs) have been shown to play a critical role in breast cancer. In recent reports, among the MMP family, gelatinases A (72-kDa gelatinase, type IV collagenase, MMP-2) and B (92-kDa gelatinase, type IV collagenase, MMP-9) play critical roles for ECM degradation and cell migration leading to tumor cell invasion in breast cancer (8,9).

MMPs are a family of zinc- and calcium-dependent endopeptidases, consisting of four subclasses based on substrate, including collagenases, gelatinases, stromelysins and membrane-associated MMPs. MMP-9 was reported to be a key enzyme for degrading type IV collagen, which is a major component of the basement membrane. Elevated MMP-9 levels are functionally linked to elevated metastasis in many tumors,

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Key words: cordycepin, matrix metalloproteinase-9, AP-1, MAPK, MCF-7 human breast cancer cells

including brain (10), prostate (11), bladder (12) and breast (13,14). Several mechanisms regulate MMP-9 activity, including gene transcription, proenzyme activation, and endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs). A variety of stimuli, including cytokines and TPA, can stimulate MMP-9 synthesis and secretion during various pathological processes such as tumor invasion, atherosclerosis, inflammation, and rheumatoid arthritis. MMP-2, on the other hand, is usually expressed constitutively (14,15). Cytokine and TPA treatments can induce MMP-9 expression via activation of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (16-18).

AP-1 is a transcription factor important in the regulation of MMP-9, as the promoter of MMP-9 gene contains binding sites for AP-1 (19). In our previous study, we reported that cordycepin inhibited MMP expressions by suppressing AP-1 activation through the MAPK signaling pathway. Therefore, it was hypothesized that cordycepin has anticancer properties inhibiting cell invasion. In this study, cordycepin was examined for its potential on TPA induced cell invasion and MMP-9 expression in breast carcinoma cells with related molecular mechanisms. Our results demonstrated that cordycepin suppresses TPA-induced MMP-9 expression by blocking the AP-1 activation via the MAPK signaling pathway and the suppression of MMP-9 expression correlates well with its inhibition of cell invasion.

Materials and methods

Cells and materials. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a 5% CO₂ incubator. Cordycepin was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). 12-O-tetradecanoylphorbol-13-acetate (TPA) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO and anti- β -actin antibodies were obtained from Sigma (St. Louis, MO, USA). Primary antibodies for p38, p-p38, JNK, p-JNK, ERK, p-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA) and MMP-9 and horseradish peroxidase (HRP)-conjugated IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). High glucose-containing Dulbecco's modified Eagle's medium (DMEM), FBS and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, MD, USA).

Determination of cell viability. The effect of cordycepin on MCF-7 cell viability was determined using an MTT assay. Briefly, cells were seeded to 3x10⁴ cells/well and allowed to attach. After 24 h, cells were treated with various cordycepin concentrations (1, 5, 10, 50 and 100 μ M). After incubation for 24 h, cells were washed with PBS, MTT (0.5 mg/ml PBS) was added to each well and the plates were incubated at 37°C for 30 min. Formazan crystals were dissolved with DMSO (100 μ l/well) and detected at 570 nm using a microplate reader (Model 3550, Bio-Rad, Richmond, CA, USA).

Western blot analysis. MCF-7 cells (5x10⁵) were pre-treated with cordycepin (50 and 100 μ M) for 1 h and then incubated

with TPA for 24 h. Cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 0.1% SDS). The protein concentration in the lysate was determined using the Bradford method (20). Samples (20 μ g) were separated by SDS-PAGE with 10% acrylamide, and transferred to Hybond™-PVDF membranes using a Western blot apparatus. The PVDF membranes were blocked with 2% bovine serum albumin or 5% skim milk, and then incubated overnight with 1 μ g/ml primary antibodies for MMP-9, p38, p-p38, JNK, p-JNK, ERK, p-ERK and β -actin. HRP-conjugated IgG was used as a secondary antibody. Protein expression levels were determined by signal analysis using an image analyzer (Fuji-Film, Japan).

Gelatin zymography assay. Conditioned media were collected after 24 h stimulation, mixed with non-reducing sample buffer, and electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 solution, and subsequently incubated at 37°C for 16 h in 5 mM CaCl₂, 0.02% Brij, and 50 mM Tris-HCl (pH 7.5). The gel was stained for 30 min with 0.25% (w/v) Coomassie brilliant blue in 40% (v/v) methanol/7% (v/v) acetic acid and photographed on an image analyzer (Fuji-Film, Japan). Proteolysis was imaged as a white zone in a dark blue field. Densitometric analysis was performed using Multi Gauge Image Analysis software (Fuji-Film, Japan).

Quantitative real-time PCR assay. Total RNA was extracted from cells using a FastPure™ RNA Kit (Takara, Shiga, Japan). The RNA concentration and purity were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 μ g total RNA using a PrimeScript™ RT reagent Kit (Takara, Shiga, Japan). MMP-9 and GAPDH mRNA expression were determined by real-time PCR using the ABI PRISM 7900 sequence detection system and SYBR® Green (Applied Biosystems, Foster City, CA, USA). The primers were: MMP-9 (NM 004994) sense, CCTGGAGACCTGAGAACCAATCT; antisense, CCACCCGAGTGTAACCATAGC and GAPDH (NM 002046) sense, ATGGAAATCCCATCACCATCTT; antisense, CGCCCCACTTGATTTTGG. To control for variation in mRNA concentration, all results were normalized to the housekeeping gene, GAPDH. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Preparation of nuclear extract. MCF-7 cells (2x10⁶) were treated with cordycepin in the presence or absence of TPA for 4 h. Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5), and pelleted at 1,500 x g for 3 min. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).

Electrophoretic mobility shift assay (EMSA). Activation of AP-1 and NF- κ B was assayed with a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ -chain (κ B, 5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3') or AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') binding site were synthesized and used as a probe for the gel retardation assay. The two complementary strands were

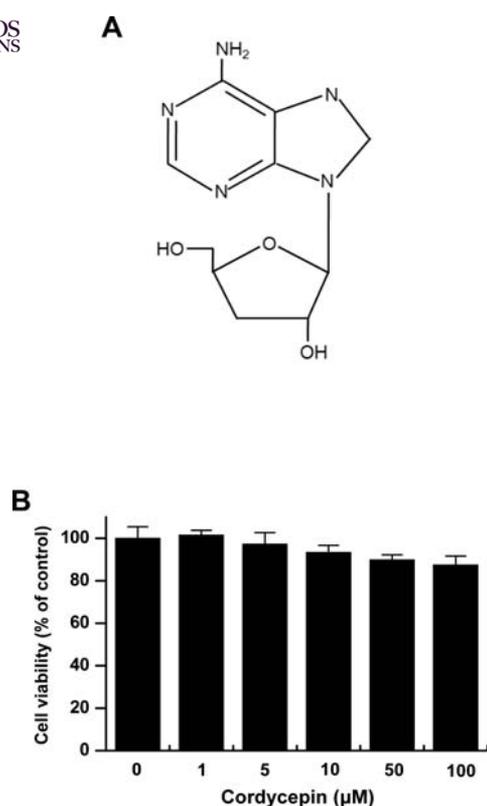


Figure 1. Structure of cordycepin and its effects on MCF-7 viability. The chemical structure of cordycepin (A). To test cytotoxicity of cordycepin, cells were cultured in 96-well plates until 70% confluence and various concentrations (1, 5, 10, 50 and 100 μM) of cordycepin were added to cells for 24 h. Methylthiazolotetrazolium (MTT) assay was used to detect the viability of the cells (B). The optical density value of control was regarded as 100%. Data represent the mean ±SEM of three independent experiments.

annealed and labeled with [α - 32 P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer [10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly (dI-dC), 1 mM DTT] were then incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB or AP-1 oligonucleotide.

Invasion assay. The invasion assay was carried out in 24-well chambers (8-μm pore size) coated with 20 μl matrigel diluted DMEM. The matrigel coating was re-hydrated in 0.5 ml DMEM for 30 min immediately before the experiments. Cells (2×10^5) were added to the upper chamber with chemoattractant in the bottom well. Conditioned medium (0.5 ml) was added to the lower compartment of the invasion chamber. The chambers were incubated for 24 h. After incubation, cells on the upper side of the chamber were removed using cotton swabs, and cells that had migrated were fixed and stained with Toluidine blue solution. Invading cells were counted in five random areas of the membrane using a light microscope. Analyzed data are the means ±SE from three individual experiments performed in triplicate.

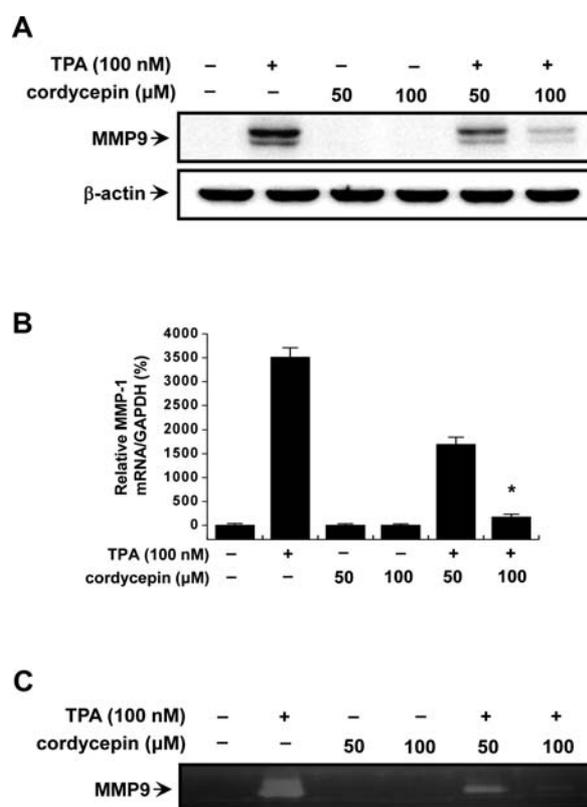


Figure 2. Cordycepin inhibits TPA-induced MMP-9 expression in MCF-7 cells. MCF-7 cells in monolayer were treated with the indicated cordycepin concentrations in the presence of TPA for 24 h. Cell lysates were analyzed by Western blotting with anti-MMP-9. The blot was reprobed with anti-β-actin to confirm equal loading (A). MMP-9 mRNA levels were analyzed by real-time PCR and GAPDH was used as an internal control (B). Conditioned medium was prepared and used for gelatin zymography (C). Each value represents the mean ±SEM of three independent experiments. *p<0.01 vs. TPA.

Statistical analysis. Statistical data analysis was performed using ANOVA and Duncan's test. Differences with a p<0.05 were considered statistically significant.

Results

Effect of cordycepin on MCF-7 cell viability. In order to investigate the cytotoxicity of cordycepin (Fig. 1A) on MCF-7 cells, the cells were seeded into 96-well culture plates at a density of 1×10^5 cells/well. Effect of cordycepin on MCF-7 cellular toxicity was analyzed using the MTT assay. Treatment of MCF-7 cells with indicated concentrations of cordycepin for 24 h did not cause any significant change in cell viability (Fig. 1B). Therefore, we performed experiments in an optimal non-toxic concentration (50 and 100 μM) of cordycepin with no change in morphology.

Effect of cordycepin on TPA-induced MMP-9 expression in MCF-7 cells. To investigate the effect of cordycepin on TPA-induced MMP-9 expression, we performed Western blot analysis, real-time PCR, and zymography in MCF-7 cells. Western blot analysis revealed that cordycepin treatment of MCF-7 cells blocked the up-regulation of TPA-induced MMP-9 protein expression (Fig. 2A). To determine the effect on TPA-induced MMP-9 secretion by cordycepin, we

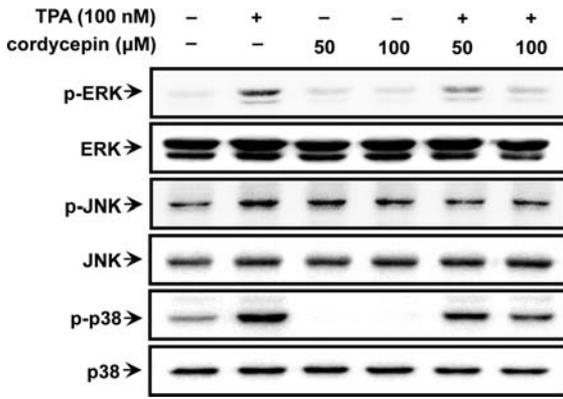


Figure 3. Cordycepin blocks TPA-induced MAPK signaling activation in MCF-7 cells. Cells were pretreated with TPA for 15 min in the presence or absence of cordycepin. Cell lysates were prepared for Western blotting with specific p-ERK, and ERK, p-p38, p38, p-JNK, JNK antibodies.

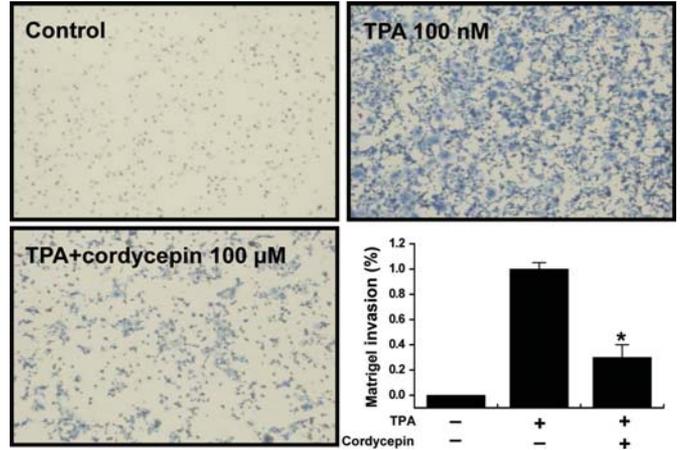


Figure 5. Effect of cordycepin on TPA-induced Matrigel invasion in MCF-7 cells. Cells were seeded onto the upper chamber and drugs were placed in the well. After a 24 h incubation, cells on the bottom of filter were fixed, stained, and counted. Each value represents the mean \pm SEM of three independent experiments. * $p < 0.01$ vs. TPA.

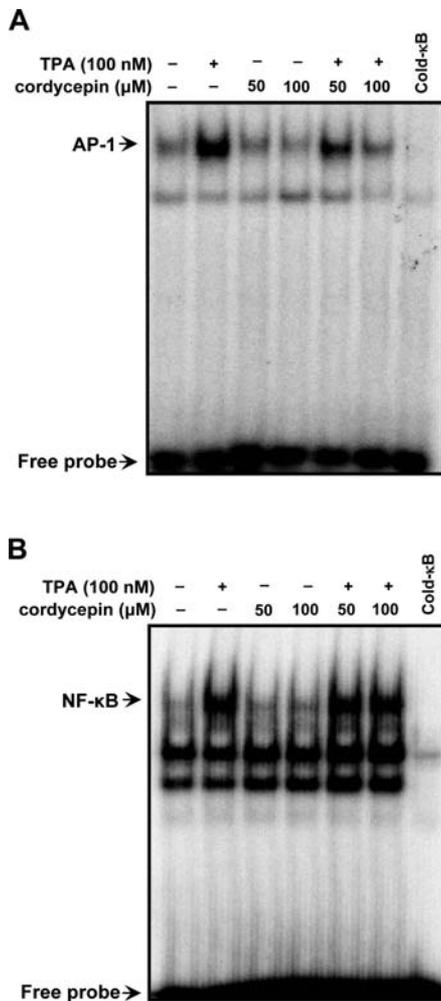


Figure 4. Cordycepin blocks TPA-induced AP-1 activation in MCF-7 cells. Cells were treated with cordycepin in the presence of TPA. Following 3 h incubation, nuclear extracts were prepared as described in Materials and methods. AP-1 and NF-κB DNA binding was analyzed by electrophoretic mobility shift analysis as described in Materials and methods (B).

performed zymography. MCF-7 cell treatment with TPA resulted in increased MMP-9 secretion. Cordycepin significantly diminished TPA-induced MMP-9 secretion (Fig. 2C). Real-

time PCR revealed that TPA increases MMP-9 level in MCF-7 cells, and that cordycepin blocked TPA-induced MMP-9 up-regulation in a dose-dependent manner (Fig. 2B). These results indicate that cordycepin is a potent inhibitor of TPA-induced MMP-9 expression in MCF-7 cells.

Effects of cordycepin on the MAPK signaling pathway by TPA. The upstream regulatory regions of MMP genes contain the AP-1 recognition site. AP-1 can be activated by the MAPK family (21,22). MAPK signaling pathway has been shown to be involved in TPA-induced MMP expression (17). To investigate which MAPK is inhibited, the effect of cordycepin on TPA-induced activation of MAPK was elucidated by using Western blot analysis. As shown in Fig. 3, treatment with TPA significantly enhanced phosphorylation of p38, JNK, and ERK. Treatment with cordycepin blocked TPA-induced phosphorylation of p38, JNK and ERK.

Effect of cordycepin on TPA-induced NF-κB and AP-1 DNA binding activities. To clarify the mechanism of cordycepin-mediated inhibition of MMP-9 expression, the effect of cordycepin on TPA-induced activation of NF-κB and AP-1 was evaluated using EMSA. As shown in Fig. 4, TPA substantially increased NF-κB and AP-1 binding activity. Treatment with cordycepin inhibited TPA-stimulated AP-1 binding activity but not NF-κB binding activity. Cordycepin itself had no effect on NF-κB and AP-1 binding activities. These results suggest that cordycepin specifically blocks AP-1 activation in MCF-7 cells.

Effect of cordycepin on TPA-induced MCF-7 cell invasion in vitro. It has been reported that the up-regulation of MMP-9 expression contributes to invasion of cancer cells (23,24). An *in vitro* invasion assay was used to investigate the inhibitory effects of cordycepin on the invasive potency of breast carcinoma MCF-7 cells. Treatment of TPA increased MCF-7 cell invasion when compared with untreated control cells, as determined by a Matrigel invasion assay. Incubation of MCF-7 cells with TPA resulted in a 10-fold increase in the invasion

 SPANDIDOS PUBLICATIONS MCF-7 cells. However, treatment with cordycepin significantly diminished the TPA-induced cell invasion by 70% (Fig. 5).

Discussion

In this study, we have for the first time provided evidence that cordycepin, a bioactive compound found in *Cordyceps militaris*, inhibits TPA-induced expression of MMP-9 in MCF-7 cells. Our results also showed that cordycepin blocked TPA-mediated activation of AP-1, but not NF- κ B, and phosphorylation of ERK, p38 and JNK.

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. This process involves cell proliferation, ECM degradation, cell migration, and tumor growth at metastatic sites (14,16). Tumor cell invasion is an early step in the metastatic cascade, representing the beginning of the transition from the benign stage to malignancy. Morphologically, tumor invasion is associated with a distorted edge of the primary tumor where individual or cohorts of tumor cells actively invade the tissue ECM surrounding the primary tumor (25).

MMPs have been regarded as major critical molecules in processing tumor invasion and metastasis. MMP-9 activation has been shown to be especially associated with tumor progression and invasion, including mammary tumors (26). In previous reports, inflammatory cytokines, growth factors, or phorbol esters were shown to stimulate MMP-9 by activating different intracellular-signaling pathways in breast cancer cells (27-29). The inhibitory effect of MMP-9 expression is important for the development of a therapeutic experimental model of tumor metastasis.

MAPK pathway is involved in the regulation of cell proliferation, apoptosis, cytokine expression, and MMP production. The three major MAPK families, JNK, ERK, and p38 kinase, are expressed and the active phosphorylated forms can be detected in MCF-7 cells (14,30). In the present study, our results suggest that cordycepin inhibits MAPK activation in TPA-mediated signaling pathways. Supporting our observations, cordycepin inhibits the phosphorylation of MAPK in macrophage cells (31). In our previous study, we reported that cordycepin inhibited MMP-1 and -3 expression by suppressing AP-1 activation through MAPK signaling pathway in rheumatoid arthritis synovial fibroblasts (32). Also, we have confirmed that TPA-induced MMP-9 expression was significantly inhibited by the selective inhibitor of ERK1/2 (PD98059) or JNK (SP600125) and partly inhibited by selective inhibitors of the p38 MAPK (SB203580) (data not shown), and previous studies support our data (30,33,34). These findings suggest that cordycepin inhibits TPA-stimulated MMP-9 expression by suppressing MAPK activation.

AP-1 and NF- κ B are transcription factors important in regulating MMP-9, as the MMP-9 gene promoter contains binding sites for both factors (19). AP-1 is a sequence-specific transcriptional factor composed of Jun, Fos, and ATF family proteins, which is induced by multiple stimuli such as TPA, cytokines, growth factors and stress (35). AP-1, which belongs to the bZIP group of DNA-binding proteins, associates to form a variety of homo- and heterodimers through a combination of

signaling events, leading to increased activity of proteins that directly potentiate Jun and Fos family members or activate transcription factors that regulate c-jun and c-fos expression. (18,36-39). NF- κ B comprises a family of inducible transcription factors which regulate host inflammatory and immune responses (40). Diverse signal transduction cascades mediate NF- κ B pathway stimulation (40). NF- κ B is an inducible dimeric transcription factor that belongs to the Rel/NF- κ B family of transcription factors and consists of two major polypeptides, p65 and p50 (41). NF- κ B is initially located in the cytoplasm in an inactive form complexed with I- κ B, an inhibitory factor of NF- κ B. Various inducers such as TPA, cytokines and stress can dissociate this complex, presumably by I- κ B phosphorylation, resulting in NF- κ B being released from the complex. NF- κ B then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription. The AP-1 and NF- κ B elements are centrally involved in MMP-9 gene induction by TPA (16,17). Previous studies have shown that the MAPK signaling pathway is important for AP-1 activation, and NF- κ B activation requires I- κ B kinase, phosphoinositide 3 kinase (PI3K)-Akt, or p38 MAPK depending on the cell type (42-46). Our results show that cordycepin inhibited MMP-9 expression by suppression of AP-1, but not NF- κ B in breast carcinoma cells.

In conclusion, our results demonstrate that cordycepin is a potent inhibitor of TPA-induced MMP-9 expression and strongly blocks the ability of MAPK/AP-1 signaling pathway in breast carcinoma cells. This is the first study showing that cordycepin suppresses TPA-stimulated cancer cell invasion by inhibiting MMP-9 expression. We also detail the molecular mechanisms of the MAPK/AP-1 pathway in breast cancer cells responsible for this inhibitory effect. Thus, cordycepin may be a potential candidate for preventing breast tumor invasion and metastasis *in vivo*.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MEST) (No. 2009-0062917), (M10528010003-05N2801-00310), the Korea Research Foundation Grant (KRF-2009-0076698), Republic of Korea, and by Korea Breast Cancer Foundation (to Hyun Zo Youn).

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