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Carnosol inhibits the invasion of B16/F10 mouse melanoma cells by suppressing metalloproteinase-9 through down-regulating nuclear factor-kappaB and c-Jun

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Abstract

Carnosol, a constant constituent of *Rosmarinus officinalis* extracts, is a phenolic diterpene shown to have antioxidant and anticarcinogen properties. In our studies, carnosol inhibited the invasion of highly metastatic mouse melanoma B16/F10 cells in vitro. First, the antimetastatic potentials of carnosol were examined by soft agar colony formation assay. Second, carnosol dose-dependently inhibited B16/F10 cell migration and invasion by in vitro transwell assay. Third, the decreasing activity of metalloproteinase was observed by zymographic assay. The result revealed that the treatment of carnosol could diminish the activity of MMP-9 more than MMP-2. Next, we analyzed the amounts of MMP-9 and MMP-2 proteins in the cells. The data indicated MMP-9 protein was also suppressed by carnosol in the same manner. In accordance with the above data, the results of reverse transcriptase polymerase chain reaction (RT–PCR) analysis showed a reduced level of MMP-9 mRNA. Furthermore, carnosol significantly inhibited the tyrosine phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, AKT, p38, JNK and inhibition of activation of transcription factors NFκ-B and c-Jun. These results lead us to conclude that carnosol could restrict the invasive ability of B16/F10 mouse melanoma cells by reducing MMP-9 expression and activity through suppressing (ERK) 1/2, AKT, p38, and JNK signaling pathway and inhibition of NF-κB and AP-1 binding activity. Taken together, these results indicate that carnosol targets MMP-mediated cellular events in cancer cells and provides a new mechanism for its anticancer activity.

Keywords: Carnosol; Matrix metalloproteinases; Metastasis; Invasion; Nuclear factor-κB (NF-κB); c-Jun

1. Introduction

Rosemary (*Rosmarinus officinalis* Labiatae, an evergreen shrub) originates from southern Europe and is one of the herbal spices of the family Labiatae [1]. In traditional herbal use, rosemary is stated to act as a mild analgesic, sedative, diuretic, antimicrobial and antibacterial agents [2]. It contains flavonoids, phenols, volatile oil and terpenoids. In the 1950s, it was reported that an extract of rosemary leaves contained high antioxidant activity. Ninety percent of this antioxidant activity can be attributed to carnosol and carnosic acid [3]. These are abundant abietane diterpenes found in the widely used Lamiaceae herbs rosemary and sage [4]. Carnosic acid is converted into carnosol by oxidation. Carnosol (Fig. 1) is a phenolic diterpene that has antioxidant, anti-inflammatory, and scavenging activities in oxidative stress models and exhibits anti-cancer activity in animal models for breast and skin tumorigenesis [5]. Carnosol inhibits dimethylbenz[a]anthracene (DMBA)-induced rat mammary DMBA–DNA adduct formation and TPA-induced ornithine decarboxylase activity in mouse skin [6], as well as lipopolysaccharide (LPS) and interferon-gamma (IFN gamma) induced nitrite production by mouse peritoneal cells [7].

Abbreviations: MMP, Matrix metalloproteinases; NF-κB, Nuclear factor-κB; AP-1, Activator protein-1; RT-PCR, Reverse transcriptase polymerase chain reaction; ERK, Extracellular signal-regulated kinase

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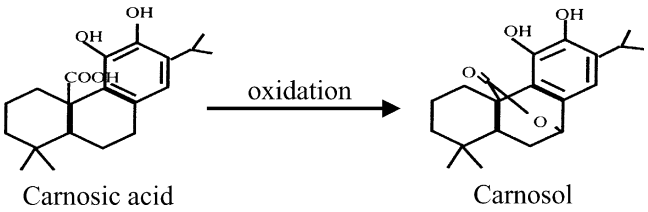


Fig. 1. The structures of carnosol.

Additional studies have revealed that carnosol strongly inhibits activities of CYP 450, a phase I enzyme, but stimulates the liver activities of glutathione *S*-transferase (GST) and NAD (P)H-quinone reductase (QR) [8]. These results give insight into the different mechanisms involved in the chemopreventive actions of carnosol. However, the antimetastatic and cytotoxic activity of carnosol has not been demonstrated until now.

Metastasis is a characteristic of high malignant cancers with poor clinical outcome. Tumor invasion is a complex cascade of events involving a finely tuned interplay between malignant cells and multiple host factors. As cancer cells become metastatic and as endothelial cells become angiogenic, they develop altered affinity and avidity for their extracellular matrix (ECM), including the basement membrane. Excess ECM degradation is one of the hallmarks of tumor invasion and metastasis. Of the several families of ECM-degrading enzymes, the most extensive are the matrix metalloproteinases (MMPs). MMPs are a large family of at least 20 zinc-dependent neutral endopeptidases that together can degrade all known components of the ECM. In addition, MMPs substantially contribute to other steps in the metastatic cascade, such as angiogenesis, differentiation, proliferation, and apoptosis [9]. Typically, MMP levels are low in normal adult tissue and become elevated only when there is a challenge to the system, such as a wound that requires repair or the onset of a disease [10]. The MMPs are overexpressed in a variety of malignant tumor types and their overexpression is associated with tumor aggressiveness and metastatic potential [11,12]. In the invasion of tumor cells, different types of MMPs are involved. MMP-1 has been observed in lung carcinomas and colorectal tumors. MMP-2 and MMP-9 found in malignant cancers, like melanoma and fibrosarcoma [13,14], are abundantly expressed in various malignant tumors and are postulated to play a critical role in tumor invasion and angiogenesis [15].

MMP-2 (gelatinase A) was first mentioned by Liotta [16]. Unlike MMP-9, MMP-2 transcriptional regulation is not readily induced by TPA or interleukin-1 (IL-1) [17]. Evidence suggests that the MMP-2 gene is a housekeeping gene and is seldom regulated at a transcriptional level. Instead, MMP-2 is regulated at a posttranslational level through interaction with TIMP-2. MMP-9 (gelatinase B) was first purified from neutral protease in neutrophils that could degrade gelatin [18]. MMP-9 protein has been studied in diverse malignant tumor cells because of its inducible character [19]. Although the two proteinases share structural and catalytic similarities, their gene expression is differentially regulated. Unlike MMP-2, which is mostly constitutively expressed, MMP-9 has a restricted pattern of expression in developmental and adult tissues and is highly regulated by many cytokines and growth factors.

Several studies have revealed that suppression of MMP-9 reduces the invasive and metastatic ability of tumor cells [20]. In previous studies, some chemicals or peptides could lower its invasive and metastatic ability by downregulating the activity and expression of MMP [21]. Several novel MMP inhibitors are being developed and some have reached clinical trails as anti-metastatic or anti-cancer therapies [22,23]. In our studies, we used B16/F10 mouse melanoma cells that have been widely used as model systems in studying metastasis behavior to demonstrate the effect of carnosol on tumor cell invasion.

2. Materials and methods

2.1. Materials

Carnosol was isolated from rosemary as described [24]. Briefly, carnosol was obtained by extracting rosemary leaves with hexane, evaporating solvent with a rotary evaporator, dissolving the dried material with methanol, and then filtrating and evaporating the solvent again. Then the dry residue was dissolved by hexane:ether (3:1) and purified by a chromatography column. The identities of the compound were confirmed by liquid chromatography/electrospray ionization mass spectrometry.

2.2. Cell culture and conditioned medium

B16/F10 cells were a gift from Prof. W.K. Yang (National Health Research Institutes, Taiwan) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL, Grand Island, NY, USA) at 37 °C under a humidified 5% CO_2 atmosphere. After a 12 h attachment, the medium was replaced with serum-free DMEM and various doses of carnosol were added. Cells were harvested and conditioned medium was collected at the indicated times.

2.3. Cell viability assay

The cytotoxicity was assayed by ATPLite^{TM}-M nonradioactive cell proliferation assay kit (Packard Bioscience B.V., Meriden, CT) using ATP as marker for cell viability according to the manufacturer's instructions. The reaction of ATP with added luciferase and D-luciferin emits light. The luminescence intensity was measured by a Topcount Microplate Scintillation and Luminescence Counter (Packard 9912v). Briefly, 3×10^4 B16/F10 mouse melanoma cells were seeded in 96 multiware. After 12 h

attachment in DMEM with 10% serum, it was replaced with serum free medium and various doses of carnosol were added. At the indicated time, cell viability was determined by ATPLite-M assay.

2.4. Soft agar colony formation assay

Effects of carnosol on the soft agar colony formation of B16/F10 mouse melanoma cells were investigated. Briefly, single-cell suspensions of B16/F10 cells were treated with or without different concentrations of carnosol, and then mixed with agarose in a final concentration of 0.35%. Aliquots of 1.5 ml containing 10^4 cells and 10% FCS were plated in triplicate in 6 cm culture dishes over a base layer of 0.7% agarose and allowed to gel. Colonies of >60 μm was counted after 14 days of incubation.

2.5. In vitro chemo-invasion assay

The migratory ability of the cells was assayed in transwell upper and lower chambers (Costar, Cambridge, MT, USA) separated by a polycarbonate membrane (8 μm pores, 6.5 mm diameter). Before invasion assays, the polycarbonate filter was coated with Matrigel (30 μg/well), a reconstituted basement membrane gel (Collaborative Biomedical Products, Bedford, MT, USA). For invasion assays, 3×10^4 B16/F10 melanoma cells were seeded in the upper well of each chamber and NIH3T3 fibroblast conditioned media was placed in the lower compartment of the chemotaxis chamber as a source of chemoattractants. After incubation for 12 h, cells were fixed with 10% formaldehyde for 10 min and stained with 0.25% Coomassie Brilliant Blue G in 45% methanol, and 10% acetic acid for 10 min. Cells in the upper chamber were removed by cotton swab. The migrated cells were counted under microscopy.

2.6. Flow cytometry

2×10^4 B16/F10 melanoma cells were cultured in 10 cm Petri dishes and incubated for 12 h. Cells were then harvested, washed with PBS, resuspended in 200 ml PBS, and fixed in 800 ml of iced 100% ethanol at −20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase), and incubated at 37 °C for 30 min. Then 1 ml of propidium iodide solution (50 μg/ml) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence that emitted from the propidium iodide–DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickenson, San Jose, CA).

2.7. Zymographic assay for metalloproteinase

The amount of gelatinase in the conditioned media (serum free) was estimated and quantified by cell numbers. Gel zymography proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in 10% polyacrylamide gels containing 0.1% (w/v) gelatin. Cell culture medium was obtained from cultured B16/F10 melanoma cells in serum-free DMEM with or without different concentrations of carnosol for 12 h in 10 cm culture dishes. Cultured media were concentrated 50-fold and mixed with Laemmeli's sample buffer in the absence of b-mercaptoethanol. Ten micro liter of sample was loaded onto the gels and electrophoresed. After PAGE, the gel was washed twice, each time for 30 min at room temperature in a solution containing 2.5% (v/v) Triton X-100 to remove SDS and subsequently transferred to a substrate buffer containing 50 mM Tris–HCl/5 mM $CaCl_2$/0.02% NaN_3, pH 8.0 at 37 °C with shaking for 24 h. Clear bands of gelatinolytic activity were visualized after staining the gel with 0.25% (w/v) Coomassie blue in 10% (v/v) acetic acid and 45% (v/v) methanol and then destaining in 10% (v/v) acetic acid and 5% (v/v) methanol.

2.8. Western blot analysis

Total cellular proteins were prepared using Gold lysis buffer containing 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 20 mM Tris-base, pH 7.9, 1 mM sodium pyrophosphate, 100 μM β-glycerophosphate,10 mM NaF; 137 mM NaCl, 5 mM EGTA, 1 mM phenylmethylsu fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cytosolic fractions and nuclear fractions were prepared according to a modified procedure of previous studies [25]. Cells were suspended in hypotonic buffer containing 10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 1.5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The nuclei were pelleted by centrifugation at 12,000 × *g* for 10 min. The supernatants containing cytosolic proteins were collected. Nuclei lysed were performed with hypertonic buffer containing 30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The supernatants containing nuclear proteins were obtained by centrifugation at 12000 × *g* for 30 min. Each lane was loaded with 30–50 μg protein separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electro-transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon^P, Millipore, Bedford, MA, USA). The membrane was pre-incubated in phosphate buffered-saline (PBS) containing 0.01% Tween-20, 1% bovine serum albumin (BSA), and 0.2% NaN_3 overnight at 4 °C and then incubated with different primary antibody followed by secondary anti-rabbit/goat/mouse IgG conjugated with horseradish peroxidase. The immunoreactive bands were visualized with enhanced chemiluminescent reagents (ECL, Amersham).

2.9. Isolation of RNA and RT-PCR

Total cellular RNA was isolated using a RNAZOL B kit (New England Biolab, Beverly, MA, USA). Total RNA (2 μg) was reverse transcribed to 10 μl cDNA with 1 mM oligo(dT)18, 0.5 mM dNTP, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 1 unit/μl RNase inhibitor, and 10 units/μl moloney murine leukemia virus reverse transcriptase at 42 °C for 1 h. The PCR reaction was proceeded in 'Ready To Go' PCR Beads (Amersham Pharmacia Biotech, New Jersey, USA). The PCR cycles were 95 °C for 1.5 min, 55 °C for 2 min, and 72 °C for 3 min. The PCR products (983-bp G3PDH fragment, 825-bp MMP-9 fragment, 762-bp MMP-2 fragment) were eletrophoresed on 1% agarose gel after 36 cycles and visualized by ethidium bromide staining. Amplification of G3PDH served as a control for sample loading and integrity. The sense and anti-sense primer sequences were G3PDH: TGAAGG TCGGTGTGAA-CGGATTGGC and CATGTAGGCCAT-GAGGTCCACCAC; MMP-9: AGGCCTCTACAGAG-TCTTTG and CAGTCCAACAAGAAAGGACG; MMP-2: GGCCATGCCATGGGGCTGGA and CCAGTCT-GATTTGATGCTTC according to previous reports [26].

2.10. Preparation of nuclear extracts and eletrophoretic mobility shift assay (EMSA)

Nuclear protein extracts (5 μg) were prepared according to the modified method of a previous study [27]. Binding activities of AP-1 and NF-κB transcription factors were analyzed by gel mobility shift assays. The cells were suspended in hypotonic buffer A: HEPES (pH 7.6) 10 mM,

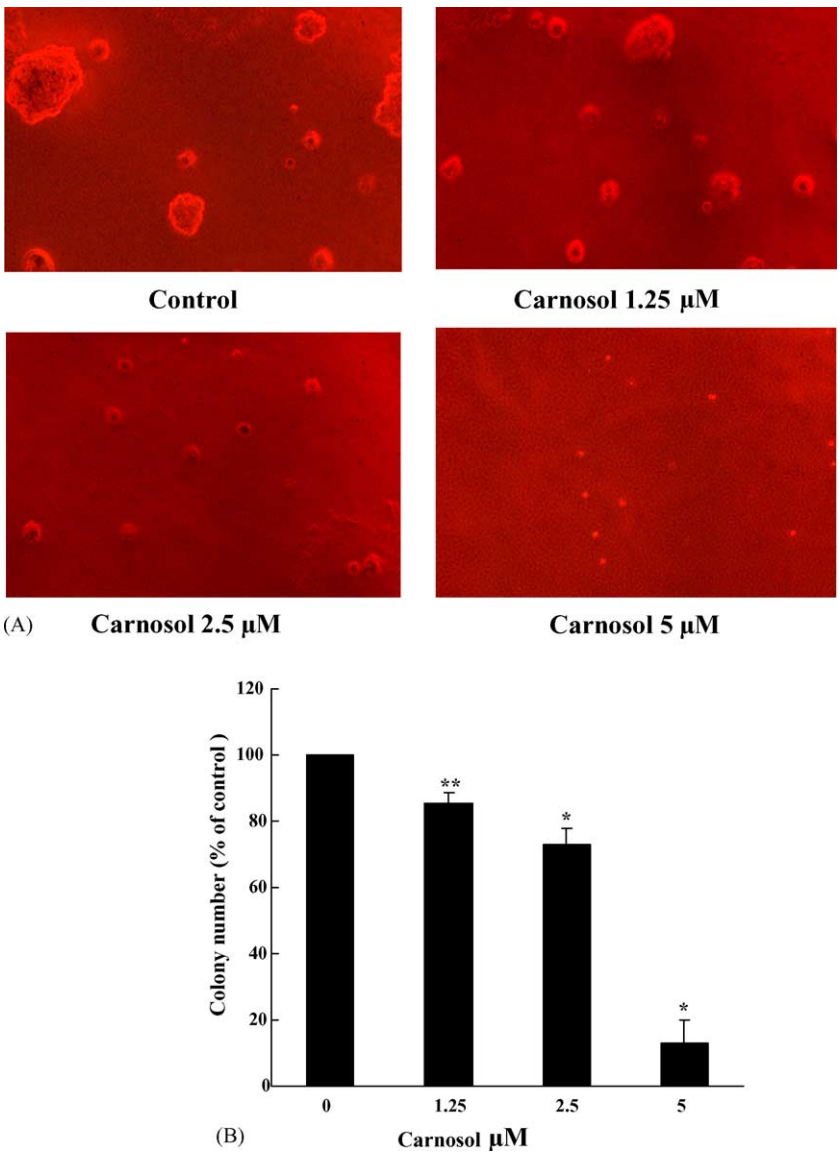


Fig. 2. Effects of carnosol on colony formation in soft agar of B16/F10 cells. (A) B16-F10 cells were treated with different concentrations of carnosol in 0.35% agarose containing 10% FCS over 0.7% agarose containing 10% FCS. Cell colonies were counted after a 14-day incubation at 37 °C in 5% CO_2 under light microscopy. (B) Colonies greater than 60 μm were scored. Statistical analysis was analyzed by *t*-test as appropriate. * Indicates the values are significantly different than the control ($^{*}P < 0.05$, $^{**}P < 0.01$).

EDTA 0.1 mM, dithiothreitol (DTT) 1 mM, PMSF 0.5 mM for 10 min on ice and vortex for 10 s. Nuclei were pelleted down by centrifugation at 12,000 × *g* for 20 s. The supernatants (cytosolic protein) were collected and the pellet was suspended in buffer C: HEPES (pH 7.6) 20 mM, EDTA 1 mM, DTT 1 mM PMSF 0.5 mM, 25% glycerol, 0.4 M NaCl, for 30 min on ice. The nuclear proteins were collected by centrifugation at 12,000 × *g* for 20 min. The probes consisted of a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), or for AP-1(5′-CGC TTG ATG ACT CAG CCG GAA-3′) endlabeled with [γ-32P]ATP using T4 polynucleotide kinase. Each nuclear extract was mixed with labeled double-stranded specific oligonucleotide, 1 μg poly (dI-dC) in the binding buffer: HEPES (pH 7.9) 25 mM, EDTA 0.5 mM, DTT 0.5 mM, NaCl 50 mM, 1% Nonidet P-40, 5% glycerol at room temperature for 20 min. The DNA/protein complexes were electrophoresed on 4.5% nondenaturing polyacrylamide gels in 5 × Tris–borate–EDTA buffer (Tris 44.5 mM, borate 44.5 mM, EDTA 1 mM), at a constant voltage of 100 V, for 3 h at room temperature. After electrophoresis, the gels were transferred to Whatman paper, dried, and exposed to X-ray film at −70 °C for 72 h.

2.11. Statistical analysis

All values were expressed as mean ± S.E. Each value is the mean of at least three separative experiments in each group. The differences in the effects of compound treatment when compared with vehicle-treated control values were analyzed by *t*-test as appropriate. * Indicates the

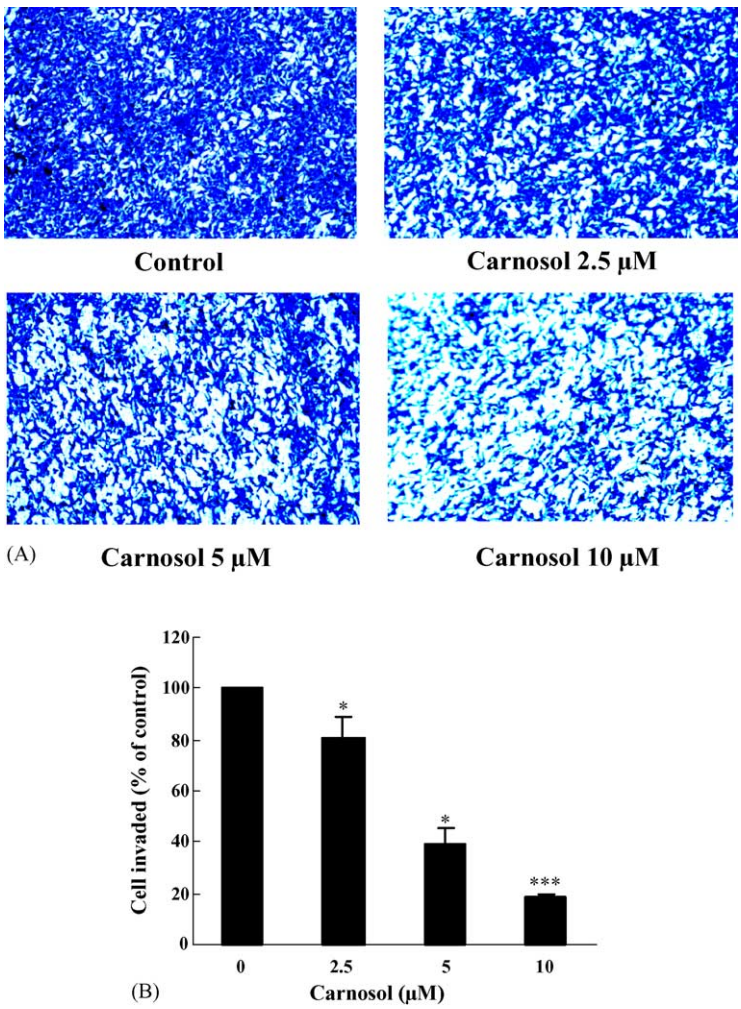
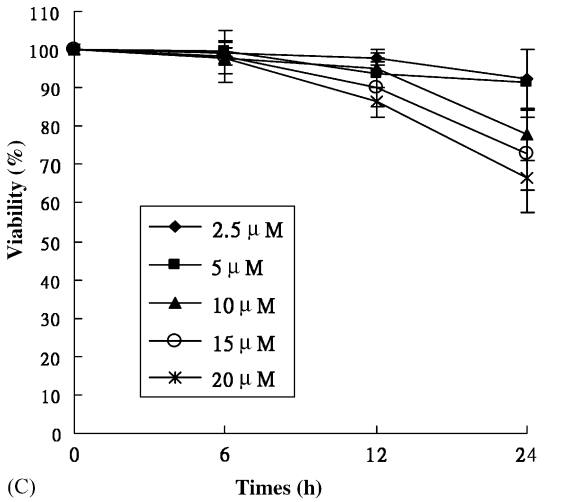


Fig. 3. Dose response of carnosol on B16/F10 melanoma cell migration. 3 × 10^4 B16/F10 melanoma cells were seeded in transwell (Coatar, Cambridge, MA, USA) upper chamber with matrigel (30 μg/well) polycarbonate membrane (8 μm pore; 6.5 mm diameter). After 12 h attachment, upper chamber was replaced with serum free media and conditioned media was added in lower chamber. At 8 h, cells on the upper surface of filter were wiped away, and stained cells on the lower surface of filter and cell migration were quantified with microscope. Panel A, representative photographs of the cells that invade Matrigel. Panel B, each bar represents mean ± S.E. of three independent experiments in triplicate. (C) Effect of carnosol on B16/F10 melanoma cells viability in serum free medium. 3 × 10^4 cells were seeded in 96 multiware. After 12 h attachment, replace with serum free medium and various dose of carnosol were added. At indicated time, cell viability was determined by ATPLite-M assay compared to the control well. Results are from one experiment representative of three similar expriments. (D) Effect of carnosol on the distribution of B16/F10 melanoma cells. Cell cycle distribution was assessed by flow cytometry. Statistical analysis was analyzed by *t*-test as appropriate. * Indicates the values are significantly different than the control ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).



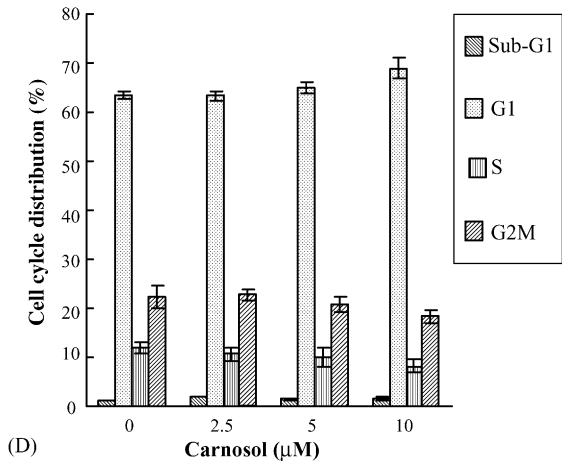


Fig. 3. (*Continued*).

values are significantly different than the control ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

3.1. Effect of carnosol on anchorage independent growth of B16/F10 melanoma cells

It has been reported that the ability of cells to form colonies in a semisolid medium is generally considered a marker of anchorage independence and is positively associated with metastatic potential [28]. Therefore, we determined the antimetastatic potential of carnosol in vitro by soft agar assay. Colonies greater than 60 μm were scored. After 2 weeks in the cultures, carnosol significantly reduced capacity for soft agar colony formation, with a lower number of colonies formed and a reduced colony size (Fig. 2A). The results of dose-dependent indicated that 5 μM of carnosol was sufficient to inhibit colony formation by 93% (Fig. 2B). The inhibition of colony formation was not due to toxicity, as determined by ATPLite-M assay (data not shown).

3.2. Inhibitory effect of carnosol on the invasion of B16/10 melanoma cells in an in vitro assay

To further evaluate the antimetastatic activity of carnosol, we assessed the inhibition of carnosol on melanoma invasion through Transwell assay. The invasive potential was determined on the basis of the cells' ability to invade a matrix barrier containing mainly laminin and type IV collagen, the major components of the basement membrane. Representative micrographs of Transwell filters are shown in Fig. 3A. B16/10 melanoma cells treated with 10 μM of carnosol for 8 h significantly inhibited the invasive activity by about 19%, respectively, compared with the control (Fig. 3B). The addition of carnosol to the B16/F10 cells led to a strong inhibitory effect on the cellular invasion. The inhibition effect of invasion was in a dose-dependent manner. In order to confirm the effect of cytotoxicity, the viability was measured. Treatment of B16/F10 cells with up to 2.5–10 μM carnosol decreased cell viability by only 10% for 12 h (Fig. 3C). However, we next questioned whether carnosol-inhibited invasion in B16/F10 mouse melanoma cells might be attributable to the cell cycle program or might have become arrested at any cell cycle phases. Our data in Fig. 3D demonstrated that carnosol-inhibited migration in B16/F10 mouse melanoma cells is not attributable to neither cell cycle program nor cell cycle attest at any cell cycle phases. Thus the data suggest that carnosol significantly reduces cell invasion but not cell viability.

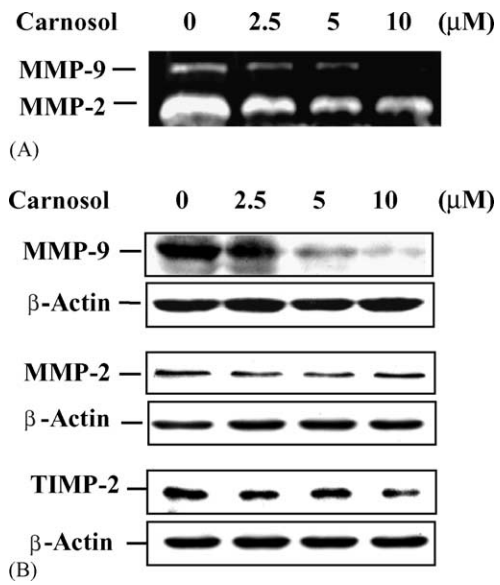


Fig. 4. Effects of carnosol on MMP-9 production in B16/F10 melanoma cells. (A) Various doses of carnosol were treated on B16/F10 melanoma cells for 12 h in serum-free medium, and concentrated aliquots of medium were electrophoresed on gelatin gels and incubated in reaction buffer as described in Section 2. (B) Effects of carnosol on the expression of MMP-9, MMP-2, and TIMP-2 in B16/F10 melanoma cell lines. Cells were treated with various concentrations of carnosol for 12 h in serum-free media. At the end of the incubation, total protein was collected and assayed by Western blot, as described in Section 2.

3.3. Effects of carnosol on the MMP-9 activity and protein level of B16/F10 mouse melanoma cells

Interestingly, MMP-2 and MMP-9 expression levels are especially high in lung carcinoma and melanoma cells [29]. These enzymes play a major role in the facilitation of cancer metastasis [30]. In B16/F10 mouse melanoma cells, invasive potential has been related to the activity and expression of MMP-9 and MMP-2 because they can degrade type IV collagen in the reconstituted basement membrane [31]. To compare the inhibition exerted on MMP-2 and MMP-9 gelatinases by carnosol, B16/F10 cell conditioned medium was analyzed by gelatin zymography in the presence of increasing carnosol concentrations. The conditioned media were collected, concentrated, and measured after 1×10^6 cells were treated for 12 h. The gelatin lysis activity in the zymogram was inhibited in a dose-dependent manner by carnosol treatment (Fig. 4A). The values of half-maximal effective inhibitory concentration (IC_{50}) for MMP-2 and MMP-9 were approximately 5 μM. In order to explore whether the down-regulation of activity was due to a diminished amount of MMP-9, we analyzed the level of MMP-9 protein in the cells. Our data revealed

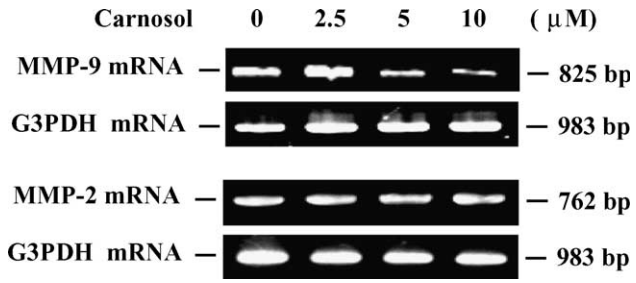


Fig. 5. Suppression of MMP-9 mRNA by carnosol. RT-PCR analysis of MMP-9, MMP-2 and G3PDH mRNA. Cells were subjected to different concentrations of carnosol for 9 h, and mRNA of MMP-9 was determined.

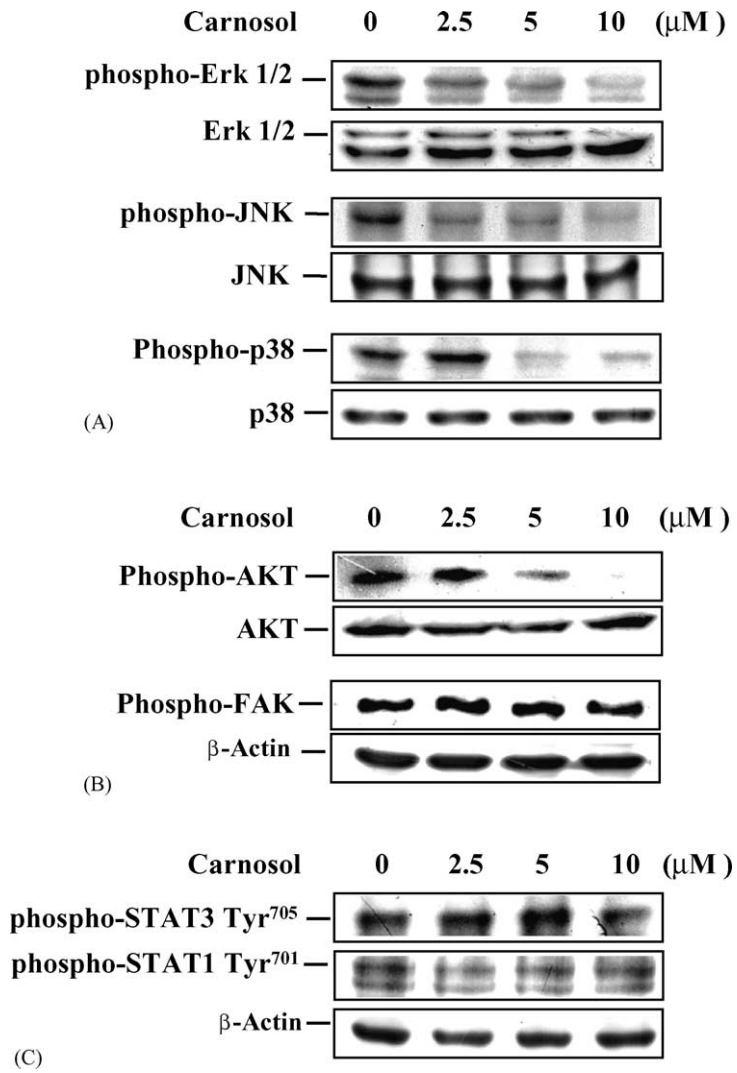


Fig. 6. Phosphorylation level of ERK1/2, p38 MAPK, JNK (A), AKT, FAK (B), STAT1, and STAT3 (C) in B16/F10 Melanoma cells treated with carnosol. Various doses of carnosol were treated on tumor cells for 6 h. The levels of activated ERK1/2, p38 MAP kinase, JNK, AKT, FAK, STAT1, and STAT3 were determined by Western blot analysis using phosphospecific antibodies. The levels of total ERK1/2, p38 MAP kinase, JNK and AKT in the same samples were also determined using anti-p38 MAP kinase, anti-JNK and anti-ERK1/2 antibodies, respectively.

that MMP-9 protein was suppressed by the treatment with carnosol. MMP-2 protein showed no significant change relative to the difference in MMP-9 protein (Fig. 4B). We next checked protein levels of TIMP-2, the endogenous inhibitors of MMP-2. TIMP-2 was only slightly affected (Fig. 4B).

3.4. Suppression of MMP-9 mRNA expression by carnosol

In order to investigate whether the suppression of MMP-9 protein by carnosol was due to reduced MMP-9 mRNA expression, RT-PCR analysis was employed on total mRNA samples extracted from B16/F10 cells after 9 h treatment. The amplification of cDNA with primers specific for mouse MMP-9, MMP-2 and G3PDH (as control gene) is shown in Fig. 5. The results indicated that lower levels of MMP-9 mRNA were expressed in the presence of carnosol in B16/F10 mouse melanoma cells, but not MMP-2. IC_{50} values for the MMP-9 mRNA levels was 5 μM, respectively. The data suggest that carnosol modulated MMP-9 expression at the transcriptional level.

3.5. Inhibition by carnosol of AKT, P38, JNK, and ERK1/2 phosphorylation

MAPKS, AKT, FAK, STAT1, and STAT3 have been shown to be involved in MMP-9 induction in various tumor types [32–34]. To examine whether the activities of MAPKs are down regulated by carnosol, we analyzed the phosphorylation of MAPKs in B16/F10 melanoma cells after treatment with carnosol (2.5–10 μM) for 6 h. Immunoblot analysis with anti-phospho-specific antibody was then performed. As shown in Fig. 6A and B, carnosol inhibited AKT, p38, JNK and ERK1/2 phosphorylation activities with a greater effect on AKT, p38, and JNK than ERK1/2. Densitometric determination indicated that the treatment of B16/F10 melanoma cells with 5 μM carnosol resulted in a 50% reduction in the amount of phosphorylated MAPKs and AKT. Carnosol did not affect FAK, STAT1, or STAT3 activity (Fig. 6C).

3.6. Carnosol reduced NF-κB and c-Jun nuclear translocation levels

Previous reports have demonstrated that the MMP-9 promoter has several transcription-factor-binding motifs, including NF-κB, AP1, and Sp1. [20]. Thus multiple pathways leading to activation of NF-κB, AP1, and Sp1-binding factors in tumor cells may contribute to MMP-9 transcription and invasion enhancement. NF-κB, AP1, and Sp1 are MAPKs and AKT-responsive promoter elements. Therefore, the MAPKs and AKT signal transduction pathway may play an important role in the regulation of MMP-9 expression. We tested whether carnosol perturbed the translocation of NF-κB, AP1, and Sp1 into the nucleus in B16/F10 mouse melanoma cells. Nucleus and cytosolic extracts were prepared and subjected to immunoblot analysis. Our data in Fig. 7A demonstrated that the amount of NF-κB (p65) and c-Jun nuclear proteins was diminished by

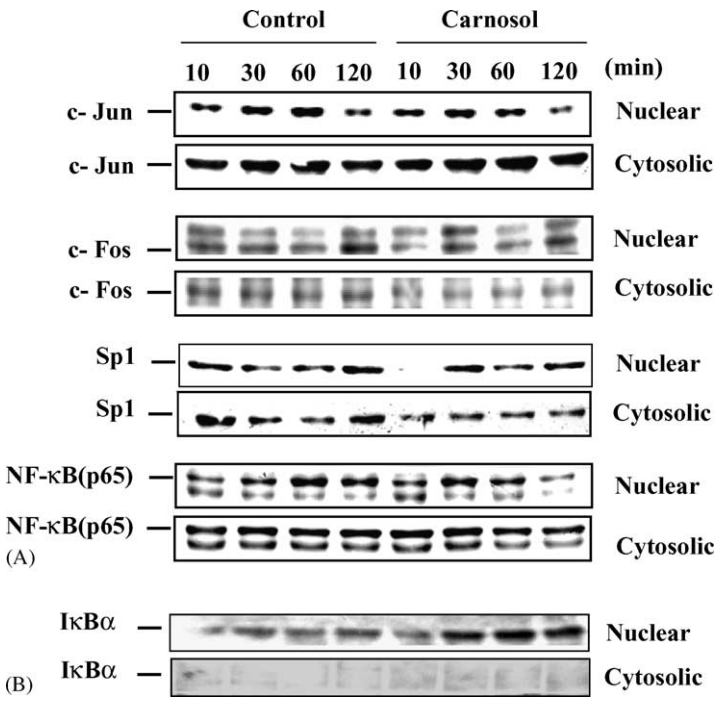


Fig. 7. (A) Effects of activated protein of c-Jun, c-Fos, Sp1, and NFκ-B by carnosol in the nucleus. (B) Carnosol prevented degradation of IκB-α. Cells were treated with 10 μM carnosol and harvested at indicated times. Nuclear and cytosolic protein extract was subjected to Western blot analysis and compared to control.

treatment with carnosol in comparison to control after 1 h. Sp1 and c-Fos nuclear translocation did not noticeably change in our range of doses. Since it has been well documented that activation of NF-κB correlates with rapid proteolytic degradation of IκB-α, prevention of IκB-αdegradation was also studied as an indication of inhibition of NF-κB activation by carnosol. As shown in Fig. 7B, the treatment of B16/F10 melanoma cells with 10 μM carnosol after 1 h prevented degradation of IκB-α.

3.7. Inhibition effect of carnosol on binding activity of AP-1 and NF-κB by electrophoretic mobility shift assay

In an additional study, electrophoretic mobility shift assay was done to confirm whether carnosol inhibited AP-1 and NF-κB binding activity in 16/F10 melanoma cells. As shown in Fig. 8, carnosol inhibited AP-1 and NF-κB transcriptional activity in a dose-dependent manner.

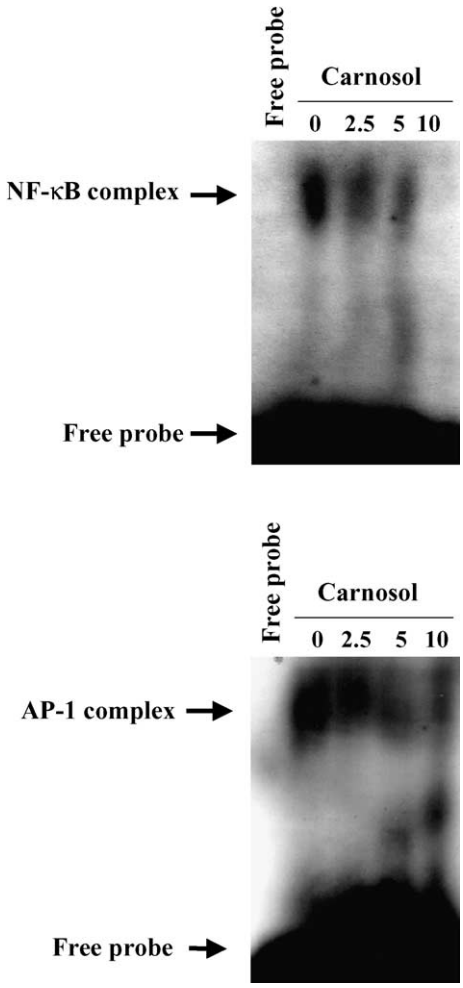


Fig. 8. Inhibition of NF-κB and AP-1 binding activity by carnosol. Nuclear extracts were prepared from B16/F10 cells treated with the indicated concentration of carnosol for 1 h. Electrophoretic mobility shift assay was carried out as described in Section 2, and the binding of nuclear extracts to [^{32}P] NF-κB and AP-1 DNA oligonucleotide is shown. The position of the NF-κB-DNA and AP-1-DNA complex is indicated with an arrow.

4. Discussion

Chemoprevention has traditionally been defined as the use of one or several natural dietary phytochemicals to prevent the occurrence of cancer [35]. Carnosol, one of the main constituents of *Rosmarinus officinalis*, believed to be among the useful chemppreventive agent. It has mainly been described as scavenging free radicals and modulating xenobiotic–metabolizing enzymes [36]. Since BHT and BHA, at high levels, have been reported to have toxicity in animals studies, the naturally occurring antioxidants in rosemary (3.02%, w/w ,of that rosemary extract was carnosol [5]) have been frequently used in certain foods instead of BHT and BHA [24]. Many studies also have demonstrated the inhibitory actions of carnosol against carcinogenesis in animal models [8,24]. Previous studies have reported that the topical application of 1, 3, or 10 μmol carnosol together with 5 nmol TPA twice weekly for 20 weeks to the back of mice previously initiated with DMBA inhibited the number of skin tumors per mouse by 38, 63, or 78%, respectively [24]. In this study, we first demonstrated that carnosol has anti-invasion activity in vitro and in vivo, and thus might represent a new strategy for cancer chemoprevention.

In recent years, attention has been drawn to the physiological relevance of MMPs, markers related to the invasive ability and malignancy of tumor cells [33,37]. As the level of expression of MMPs increases, the invasive ability also increases. Studies performed over several decades have suggested that growth factors and cytokines secreted by tumor cells will induce the production of MMPs. Transcriptional activity plays a crucial role in the regulation of MMP expression. Several studies have identified signal transduction pathway involved in the regulation of MMP-9 expression in tumor cells [33]. A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves activation of kinases. These kinases, serine/threonine kinases related to the mitogen-activated proteins kinase (MAPK) superfamily, mediate signals from cell membrane receptors triggered by growth factors, cytokines, and cell-matrix interactions. MAPKs are intricately involved in the expression of the components involved in MMP-9 promoter induction via NF-κB, AP-1, and its association with c-Jun and c-Fos. At least three subgroups of MAPK family members have been implicated: extracellular signal-regulated kinases (ERKs), c-Jun *N*-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38MAPK [38]. Additionally, the PI3K and AKT signal pathways also play a crucial role in MMP-9 gene regulation [39]. Here, we used a compound, carnosol, to further investigate in detail the mechanisms of the effect on MMP gene expression.

In the present study, carnosol inhibited the invasion of metastatic B16/F10 mouse melanoma cells into a reconstituted basement membrane gel in a concentration dependent manner (Fig. 3). In the range of concentrations tested,

the inhibitory effect on invasion was clearly observed at 8 h without an effect on the viability of the cells (93% until 12 h). When treating with 10 μM carnosol or even a higher dose (20 μM), until 24 h, the percentage of viable tumor cells was reduced to 65–75% (Fig. 4A). The rate of cell growth and viability were also affected by the treatment at longer times or higher doses. The results showed, during the short time interval (<12 h), that the diminished invasion ability of cells was not due to reduce growth. The data are consistent with the previously drawn conclusions. The inhibitory effect of carnosol on tumor invasion may be associated with the degradative cascade of ECM and basement membrane. The enzymatic activities of type IV collagenase (MMP-2 and MMP-9) and the expression of their mRNAs correlate especially well with tumorigenicity and the metastatic ability of tumor cells [40]. The activity of MMP-9 and MMP-2 was measured because they can degrade type IV collagen in the reconstituted basement membrane. Zymographic analysis in Fig. 4A showed that carnosol was able to inhibit the gelatinolysis mediated by MMP-2 and MMP-9 in a conditioned medium of metastatic B16/F10 cells at 12 h. Also, the activity of MMP-9 was more clearly suppressed than that of MMP-2. Carnosol was able to inhibit the production of MMP-9 from tumor cells in a concentration-dependent manner, though MMP-2 did not change (Fig. 4B). It has recently been reported that EGCG preferentially binds to MMP-2 and MMP-9 resulting in the formation of EGCG–MMP complex [21,41]. The exact molecular mechanisms by which EGCG blocks gelatinolytic activities remain largely unknown. It is not clear whether carnosol is able to directly inhibit the activity of the active form of MMP or the activation/conversion of the latent form to the active form of MMP. Thus, the detailed inhibitory mechanism is not yet determined, but these results clearly indicate that the anti-invasive effect of carnosol is associated with inhibition of the enzymatic degradative processes of tumor invasion. In order to evaluate whether the reduction of the lytic content of MMP-9 is due to a decrease in the amount of corresponding MMP-9 mRNA, the efficacy of MMP-9 gene transcription was detected by RT-PCR analysis. The dose-dependent responses suggest that carnosol has the ability to inhibit the invasion of tumor cells by diminishing the efficiency of gene transcription (Fig. 5).

Regulation of MMP-9 activation by various stimuli in different cellular settings may involve different signal transduction pathways. Binding sites for both activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) transcription factors exist in the conserved regions of the rabbit MMP-9 gene promoter [42]. In addition, several growth factors and cytokine regulatory pathways converge at the AP-1 and NF-κB binding site, including EGF, TPA, IL-1, TNF-α, and LPS [43]. MMP-9 activity was markedly stimulated in tumor cell lines by EGF, a known MAPK and PI3K activator, suggesting a possible role for MAPK and PI3K in the activation of MMP-9 [39]. It has been reported that EGF-induced MMP-9 protein expression involves both PI3K and MAPK signaling in the ovarian carcinoma cell line OVCA 429 [32]. In addition, PMA-induced MMP-9 activities in the UM-SCC-1 cell line were abolished by inhibition of p38 kinase [44]. Inhibition of ERK activation by MEK inhibitor PD098059 blocked MMP-9 production and attenuated the in vivo invasiveness of head and neck squamous carcinoma cells [45]. Involvement of JNK and PI3K signaling pathways in regulation of MMP-9 was also reported [19]. Our previous studies [2] have indicated that carnosol inhibited NO synthase (iNOS) and NF-κB promoter activity induced by LPS through suppressing p38 and ERK signaling pathway. Thus, the action mechanism of carnosol, which inhibited metastasis might work through those pathways. Our work provides insight on how carnosol suppressed MAPKs and the AKT signaling pathway and reduces AP-1 and NF-κB transcriptional activity in B16/F10 mouse melanoma cells. The AP-1 binding site on the MMP-9 promoter was shown to play a master role in the induction of MMP-9, cooperating synergistically with the element of the NF-κB-like and SP-1-like factors [46]. Indeed, the inhibitory effect of carnosol on MMP-9 transcriptional activity was through inhibition of NF-κB and AP-1 binding activity in our data. The signal to the AP-1 sites is common for the genes of TPA-inducible MMPs such as MMP-1, MMP-3, and MMP-7 [47], which suggests that carnosol may inhibit their expression.

Elevated expression of MMP-9 is associated with increased metastatic potential in many cancer types, including breast, prostate, and brain cancer, and melanoma [48]. The activity and expression of MMP-9 are regarded as metastatic markers in transformed cell lines. Carnosol inhibition of MMP-9 gene expression is demonstrated in this paper for the first time. The results above imply that carnosol strongly inhibits lung metastasis of B16/F10 melanoma cells by inhibition of NF-κB and AP-1 binding activity. Then, it reduces the transcription of MMP-9 mRNA and the translation of MMP-9 protein, thereby decreasing the activity of MMP-9 in the cells. These results highlight the potential of carnosol in the treatment of cancer metastasis.

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