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Cryptotanshinone from *Salvia miltiorrhiza* BUNGE has an inhibitory effect on TNF-α-induced matrix metalloproteinase-9 production and HASMC migration via down-regulated NF-κB and AP-1

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ABSTRACT

Matrix metalloproteinases (MMP-9 and MMP-2) production and smooth muscle cell (SMC) migration may play key roles in the phathogenesis of neointima formation and atherosclerosis. Especially inducible MMP-9 expression was directly involved in the cancer cell invasion and SMC migration through vascular wall. In this study, we reveal that cryptotanshinone (CT) purified from *Salvia miltiorrhiza* BUNGE had an inhibitory effect on MMP-9 production and migration of human aortic smooth muscle cells treated with TNF-α in a dose-dependent manner. The down regulation of transcription of MMP-9 mRNA was evidenced by RT-PCR and MMP-9 promoter assay using luciferase reporter gene. Eletrophoretic mobility shift assay showed NF-κB and AP-1 nuclear translocations were suppressed. In addition, Western blot analysis indicated that extracellular signal regulated kinase 1 and 2, p38 and JNK MAP kinase signaling pathways were inhibited. From the results, it is suggested that CT has anti-atherosclerosis and anti-neointimal formation activity.

1. Introduction

In the past several years, a number of studies have demonstrated that matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9, are important for smooth muscle cell (SMC) proliferation and migration into the intima [1]. An increased proteolytic activity in the vessel wall mediates the degradation of the extracellular matrix surrounding SMCs in response to injury [1], a necessary step for permitting medial SMCs to migrate into the intimal space. One class of MMPs that has been implicated as mediator of lesion development in response to vascular injury is the gelatinases, MMP-2 (72 kDa)

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and MMP-9 (92 kDa). In a rat arterial injury model, MMP-9 is expressed within 6 h after injury in rat carotid arteries and continues to be expressed for up to 6 days, whereas, MMP-2 activity is substantially increased 4 days after injury [2]. Recent reports from a knockout study concluded that MMP-9 is critical for the development of arterial lesions via its ability to regulate both vascular smooth muscle cells (VSMC) migration and proliferation [3,4]. Although MMP-2 and MMP-9 have similar substrate specificities [5,6], there are differences in the regulation of their expression. MMP-2 is constitutively expressed by several cell types, including smooth muscle cells, and its expression is not induced by cytokines or growth factors [7,8]. In contrast, based on reports from several different laboratories, it has generally been concluded that the basal levels of MMP-9 are usually low, and its expression can be induced by treatment of cells with tumor necrosis factor-α (TNF-α), but not platelet-derived growth factor (PDGF) or thrombin [7–9]. Studies of signal transduction pathways that regulate MMP-9 expression have demonstrated that the activation of the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and PI-3 kinase, is critical for the increased expression of MMP-9 in response to different agonists [9–11]. It has been shown that in VSMC, TNF-α and nerve growth factor (NGF) regulates MMP-9 expression through the activation of ERK1/2 [9,11].

The endogenous MMP inhibitors, known as tissue inhibitors of metalloproteinase (TIMP), maintain a balance between matrix formation and destruction but have short half-lives [12]. Therefore, synthetic inhibitors of MMPs, such as BB94 (batimastat) and BB2516 (marimastat), have been developed. BB94 has a structure that mimics collagen and facilitates chelating of the zinc ion in the active site of the MMP molecule, thereby causing an inactive protease [13]. During the last few years, substances of this type have been used to study the importance of extracellular matrix (ECM) remodeling in various types of diseases, including rheumatoid arthritis, cancer invasion, and cardiovascular diseases. Thus, synthetic MMP inhibitors have been demonstrated to affect the wound-healing response in injured rat carotid arteries [14,15], to reduce migration of VSMC from baboon aortic medial explants [16], and to inhibit the macrophage modulation of phenotypic change and DNA synthesis in cultured rabbit aortic SMC [17].

The abnormal growth of VSMC and ECM proteins are prominent features of vascular disease, including atherosclerosis, restenosis after angioplasty [18]. Atherogenic lesions form during several pathological processes, which involve the accumulation of inflammatory cells and the release of cytokines [19]. TNF-α is a cytokine secreted by VSMC in the neointima after balloon-injury as well as by macrophages in atherosclerotic lesions [20–22]. TNF-α induces the activation of ERK1/2, a key transducer of extracellular signals that promote cell growth and movement, which are critical for the initiation and progression of vascular lesions [23,24].

Several natural products have been used for vascular diseases [25], and some traditional herbal prescriptions have been also employed for treatment of atherosclerosis [26,27]. *Salvia miltiorrhiza* BUNGE is a well-known traditional medicine used for the treatment of cardiovascular diseases in oriental countries. Recent studies have showed that *S. miltiorrhiza* BUNGE significantly inhibited intimal hyperplasia and attenuated restenosis [28]. In addition, the CT has an anti-angiogenic activity in basic fibroblast growth factor (bFGF)-treated BAECs [29]. The CT is one of the major constituents purified from *S. miltiorrhiza* BUNGE and we have hypothesized that CT may inhibit the thickening of intima after denudation through inhibition of MMPs activity, which is responsible for migration of VSMC from media to intima. Therefore, we carried out this study to elucidate the inhibitory potential of CT on HASMC migration and MMP-9 production induced by TNF-α treatment.

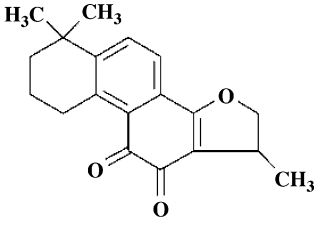
2. Materials and methods

2.1. Materials and cell cultures

The CT was dissolved in dimethyl sulfoxide (DMSO). Recombinant human TNF-α was obtained from R&D systems (Boston, MA). HASMC were our deposit [30], which was purchased from Bio-Whittaker (California, USA). HASMC were cultured in smooth muscle cell growth medium-2 containing 10% fetal bovine serum (FBS), 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 50 μg/ml gentamicin, 50 μg/ml amphotericin-B, and 5 μg/ml bovine insulin. For all experiments, early passage HASMC were grown to 80–90% confluence and made quiescent by serum starvation (0.1% FBS) for at least 24 h. The serum-free medium contained secreted proteins, such as MMP-9. The amount of secreted proteins in the conditioned media was estimated and quantified by cell numbers.

2.2. Preparation of CT from S. miltiorrhiza BUNGE

The dried callus was powdered and extracted with methanol (100 ml) twice. Methanol soluble fractions were concentrated under vacuum and the obtained residue (1.25 g) was subjected to chromatography. Column chromatography was performed on a 5350-cm column packed with silica gel 60 (finer than 200 and 100–200 mesh, Merck). The column was packed with benzene, and the sample was loaded in benzene, and then eluted with benzene, followed by consecutive elution with benzene containing ethyl acetate 2%, 5%, 8%, 10%. Fractions eluted with 8% ethyl acetate were concentrated. Further purification through column chromatography followed by preparative TLC (benzene:ethyl acetate; 8:2) resulted in an orange red solid (20 mg). Structure of the compound (Fig. 1) was established based on NMR and mass spectral data and by comparison with those of authentic sample.



CT: $C_{19}H_{18}O_3$(294.33)

Fig. 1 – The chemical structure of CT from *Salvia miltiorrhiza* BUNGE.

2.3. Western blot analysis

Quiescent HASMC were treated in the presence or absence of TNF-α as indicated. To detect MMP-9 and GAPDH protein in Western blot analysis, cells were homogenized in a sample buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 1% Triton X-100. Protein concentrations were measured using the Bio-Rad protein assay, and blotted membranes were incubated with the MMP-9 (Serotec, Kidlington, Oxford, UK) and GAPDH antibodies (Chemicon, El Segundo, CA, USA). The monoclonal antibodies and polyclonal antibodies to p-ERK1/2, p-SAPK/JNK, and p-p38 were purchased from New England Biolabs (Beverly, MA). [γ^{32}]P-ATP was obtained from Amersham (Buckinghamshire, UK). Detection was performed using a secondary horseradish peroxidase-linked anti-mouse antibody and the ECL chemiluminescence system (Amersham, Arlington Heights, IL, USA).

2.4. Promoter assay

A 710 bp fragment from the 5′-promoter region of the MMP-9 gene was cloned. A 710 bp fragment at the 5′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (accession no. D10051): 5′-ACATTTGCCCGAGCTCCTGAAG (forward/*Sac*I) and 5′-AGGGGCTGCCAGAAGCTTATGGT (reverse/*Hin*dIII). The pGL2-basic vector containing a polyadenylation signal upstream from the luciferase gene was used to construct the expression vectors by subcloning PCR-amplified DNA of the MMP-9 promoter into the *Sac*I/*Hin*dIII site of the pGL2-basic vector (WT-MMP9 pro). PCR products (fragment of MMP-9 promoter) were confirmed by their size, as determined by electrophoresis, and by DNA sequencing. Cells were plated onto six-well plates at a density of 10^5 cells/well and grown overnight. Cells were cotransfected with 1 μg of MMP-9 promoter-luciferase reporter constructs and 1 μg of β-galactosidase reporter plasmid by the LipofecAMINE method (Invitrogen, San Diego, CA, USA). Cells were cultured in 10% FBS medium and incubated with drugs for 24 h. Luciferase activity and β-galactosidase activity were assayed by using the luciferase and β-galactosidase enzyme assay system (Promega). Luciferase activity was normalized with the β-galactosidase activity in the cell lysate and calculated as an average of three independent experiments.

2.5. Electrophoretic mobility shift assay (EMSA)

The nuclear extract of each cell was prepared as described below. Cells were washed with cold PBS and suspended in 0.4 ml of lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin. The cells were allowed to swell on ice for 15 min, and then 25 μl of 10% Nonidet P-40 was added. The tube was vigorously vortexed for 10 s, and the homogenate centrifuged at 4 °C for 2 min at 13,000 rpm. The nuclear pellet was resuspended in 50 μl of ice-cold nuclear extraction buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin. The tube was incubated on ice for 15 min with intermittent mixing. The nuclear extract was then centrifuged at 4 °C for 5 min at 13,000 rpm and the supernatant was either used immediately or stored at −70 °C for later use. The protein content was measured using the Bio-Rad protein assay. EMSA were performed using a gel shift assay system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequences for AP-1 (5′-CTGACCCCTGAGTCAG-CACTT-3′), and NF-κB (5′-CCAGTGGAATTCCCCAG-3′) were end-labeled with [γ-^{32}P] ATP (3000 Ci/mmol; Amersham Pharmacia Biotech) using T4 polynucleotide kinase and used as probes for EMSA. Competition was performed using either the unlabeled AP-1, NF-κB. oligonucleotides. Nuclear extract proteins (2 μg) were preincubated with the gel shift binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 0.05 mg/ml poly-deoxyinosine–deoxycytosine) for 10 min, then incubated with the labeled probe for 20 min at room temperature. Each sample was electrophoresed in a 4% nondenaturing polyacrylamide gel in 0.5× TBE buffer at 250 V for 20 min. The gel was dried and subjected to autoradiography.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

To detect the expression of MMP-9 using RT-PCR, total RNA was prepared from HASMCs using the RNAzol B reagent (Teltest, Friendswood, TX, USA) according to the manufacturer's instructions. For RT-PCR, a cDNA was synthesized from 1 μg of total RNA using AMV RNA PCR Kit (Takara, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: MMP-9 (537 bp), 5′-CGGAGCACG-GAGACGGGTAT-3′ (sense) and 5′-TGAAGGGGAAGACGCA-CAGC-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCA-CGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

2.7. Cell viability assay

The cytotoxic effect of the CT on HASMC was investigated using a commercially available proliferation kit (XTT II, Boehringer Mannheim, Mannheim, Germany). Briefly, the cells were plated in 96-well culture plates at a density of 5×10^3 cells/well in DMEM culture medium and allowed to attach for 24 h. After incubation the medium were discarded and replaced with 100 μl of new medium containing various concentrations of CT. After 24 h of culture, 50 μl of XTT reaction solution (sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and *N*-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the wells. The optical density was read at 490 nm wavelength in an ELISA plate reader after 4 h incubation of the plates with XTT in an incubator (37 °C and 5% CO_2 + 95% air). All determinations were confirmed using replication in at least three identical experiments. The data were shown mean ± S.E. as percent of control.

2.8. Invasion assays

Matrigel invasion assay was performed as described previously [31]. Briefly, Matrigel-coated filter inserts (8 μm pore size) that fit into 24-well invasion chambers were obtained from Becton–Dickinson (New Jersey, USA). HASMC (5×10^4 cells/well) to be tested for invasion were detached from the tissue culture plates, washed, resuspended in conditioned medium collected from without-TNF-α or TNF-α-treated or CT (0, 5 and 10 μg/ml) + TNF-α treated to HASMC for 24 h, and then added to the upper compartment of the invasion chamber. Five hundred microliters of the same conditioned medium was added to the lower compartment of the invasion chamber. Cells without TNF-α-treated conditioned medium served as control. The Matrigel invasion chambers were incubated at 37 °C for 24 h in 5% CO_2. After incubation, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, mounted, and stained according to the manufacturer's instructions. The cells that invaded through the Matrigel and were located on the underside of the filter were counted. Three to five invasion chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters.

2.9. Gelatin zymography assay

Gelatin zymography was performed as described previously [32] with some modification. Culture supernatants of HASMC treated with or without TNF-α (100 ng/ml) were resuspended in a sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue and loaded without boiling in 7.5% acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin. Electrophoresis was carried out at a constant voltage of 100 V. After electrophoresis, the gels were soaked in 0.25% Triton X-100 (twice for 30 min) at room temperature and rinsed in distilled pure water. For inhibitory effect of CT on TNF-α induced MMP-9 expression, HASMC were treated with various concentrations of CT in the presence of 100 ng/ml TNF-α and MMP-9 expression was evaluated by zymography. HASMC were grown in 10% FBS/DMEM and rinsed with PBS, then incubated in serum-free DMEM with or without drug (CT) in the presence of TNF-α for 24 h and the conditioned media were collected. The conditioned medium was resolved in 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed for 1 h in 2.5% (v/v) Triton X-100 to remove SDS, and then incubated for 24 h at 37 °C in the incubation buffer to allow proteolysis of the gelatin substrate. Bands corresponding to activity were visualized by negative staining using Coomassie Brilliant blue R-250 (Bio-Rad, Richmond, CA, USA) and molecular weights were estimated by reference to prestained SDS-PAGE markers.

2.10. Image analysis

The intensity of the bands obtained from zymogram studies and Western blot images was captured and estimated with Scion Image (Scion, MA, USA).

3. Results

3.1. CT inhibits the MMP-9 activity

As reported previously, it is known that MMP-9 is highly induced by TNF-α (100 ng/ml) in VSMC [30,33,34] and plays an important role in regulating a variety of biological functions. In this study, we examined the effect of CT on the production of MMP-9 in TNF-α stimulated HASMC. Treatment of HASMC with TNF-α caused a substantial increase in the production of MMP-9. CT inhibited the secretion of MMP-9 that is induced by TNF-α. The HASMC were incubated with serum free media to induce quiescence and CT was added to the media in 1 or 2 h before treatment of TNF-α. After further incubation for 24 h, the media was harvested and introduced to zymography. The production of MMP-9 was inhibited by CT in a dose-dependent manner (Fig. 2A). In order to confirm the MMP-9 protein decrease we performed Western blotting, when the same amount of total cellular protein was introduced to blot, the MMP-9 protein level was decreased (Fig. 2B). Additionally

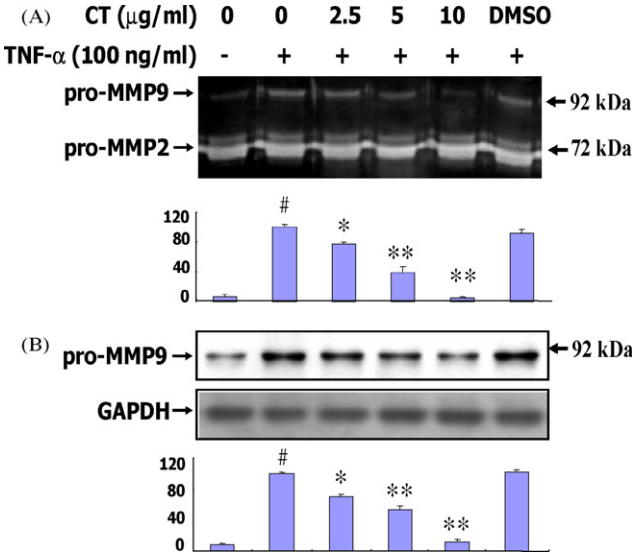


Fig. 2 – CT inhibits MMP-9 production (A) decreased MMP-9 was detected in zymography. In HASMC culture, the media was changed with serum free DMEM at 70–80% confluence and the CT were treated to culture media dose dependently (2.5, 5, and 10 μg/ml) and 1 h later 100 ng/ml TNF-α was treated. In zymography, 24 h incubated media was used to find secreted MMP-9 protein. MMP-9 level was suppressed at 10 μg/ml CT concentration. The DMSO (10 μl/ml) was used as an inert control because it was used as vehicle solution dissolving CT and the treated amount was 2.5 times excess than 10 μg/4 μl of CT's stock solution. (B) Western blotting revealed the decreased MMP-9 protein when the same amount of total cellular protein was used. The data represent the mean ± S.D. of intensity ratios (MMP-9/GAPDH) at least three independent experiments. Results were statistically significant ($^{\#}p < 0.05$ compared with control, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with TNF-α only stimulation, respectively by using Student's t-test).

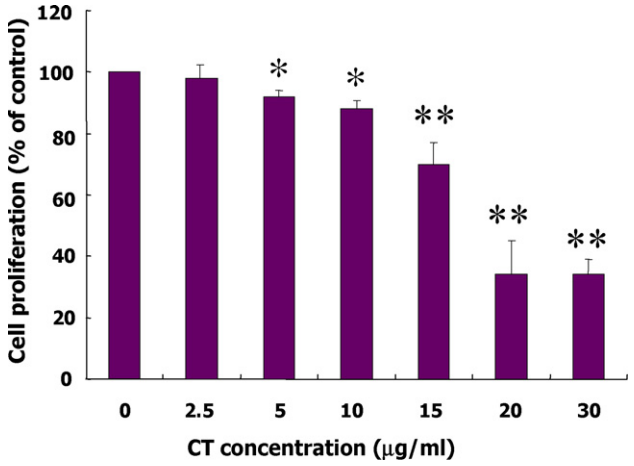


Fig. 3 – CT inhibits cell proliferation. HASMC was plated in 96-well plate and the media was changed with serum free DMEM at about 70–80% confluence, and then CT were treated as 2.5, 5, 10, 15, 20 and 30 μg/ml concentrations. The XTT solution was added at 20 h later. After 24 h the 490 nm wavelength was measured. Above 10 μg/ml concentration of CT significantly suppressed the HASMC proliferation. The results are expressed as the mean ± S.D. of three independent experiments. Statistical significance was determined ($^{*}p < 0.05$; $^{**}p < 0.01$ compared with control) using Student's t-test.

the media collected from TNF-α stimulated HASMC was treated with CT for 24 h, but the MMP-9 activity was not affected.

3.2. *Cytotoxicity of CT on HASMC cells*

The cytotoxity of CT on the HASMC cells was evaluated using XTT cell proliferation assay kit. The HASMC cells (5×10^3 cells/well) were incubated for 24 h in cultures in 96-well microplates with various final concentrations of CT (0, 2.5, 5, 10, 15, 20 and 30 μg/ml). Dose-dependent cytotoxic effect of CT against HASMC was shown in Fig. 3. We determined proper concentrations of CT for treatment. Ten micrograms per milliliter concentration of CT has a weak cytotoxic effect on HASMC and used in this study.

3.3. *Effect of CT on transcriptional activity of MMP-9 promoter in TNF-α stimulated HASMC*

Our finding that CT inhibits TNF-α-stimulated MMP-9 expression in HASMC as measured by Western blot analysis prompted us to investigate the effect of CT on MMP-9 promoter activity and levels of MMP-9 mRNA. To investigate the effect of CT on transcriptional levels of MMP-9 mRNA, we performed RT-PCR analysis to evaluate MMP-9 mRNA levels. The levels of MMP-9 transcript were down-regulated as shown in Fig. 4B. For further confirming of transcriptional inhibition of CT to MMP-9 expression, we performed a transient transfection and luciferase reporter assay. The wild type MMP-9 promoter region (−710 and −29-bp upstream fragment

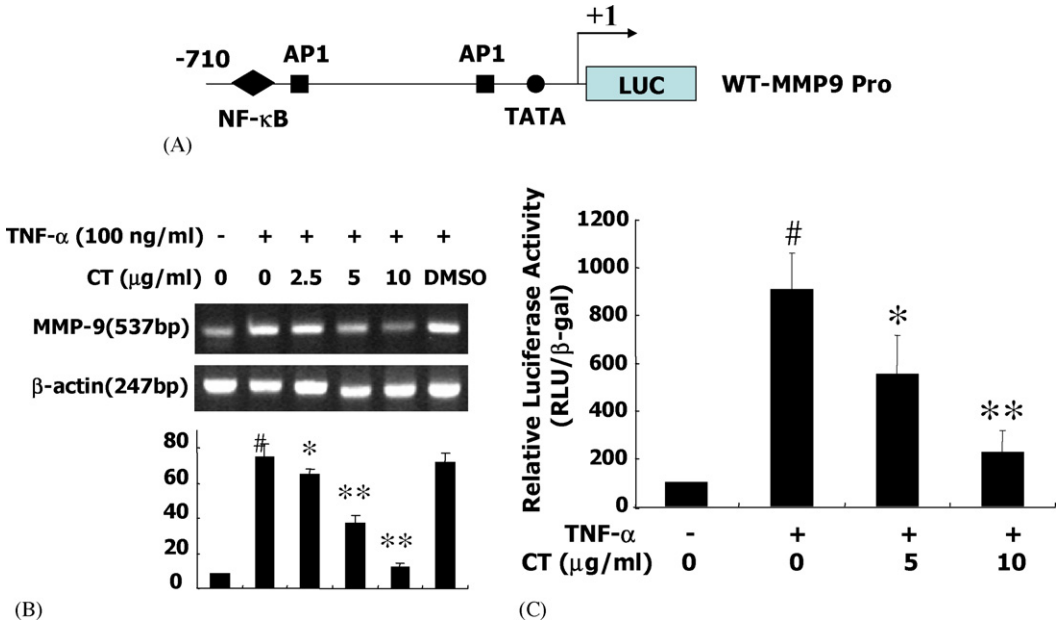


Fig. 4 – MMP-9 mRNA level was transcriptionally down regulated. (A) MMP-9 promoter region construction with luciferase reporter gene. (B) RT-PCR with MMP-9 specific primers. The amplified DNA fragment was downed when CT treated. The 10 μl/ml DMSO was used as an inert control. The bars are represented as the mean ± S.D. of the ratios (MMP-9/beta-actin) from three independent experiments. (C) Luciferase activity was measured in transiently transfected HASMC with pWT-MMP-9 pro and the promoter activity was blocked in 10 μg/ml CT concentrations. Values represent the mean of at least three independent experiments; bars: ±S.D. Statistical significance was determined ($^{\#}p < 0.05$ compared with control, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with TNF-α only stimulation, respectively by using Student's t-test).

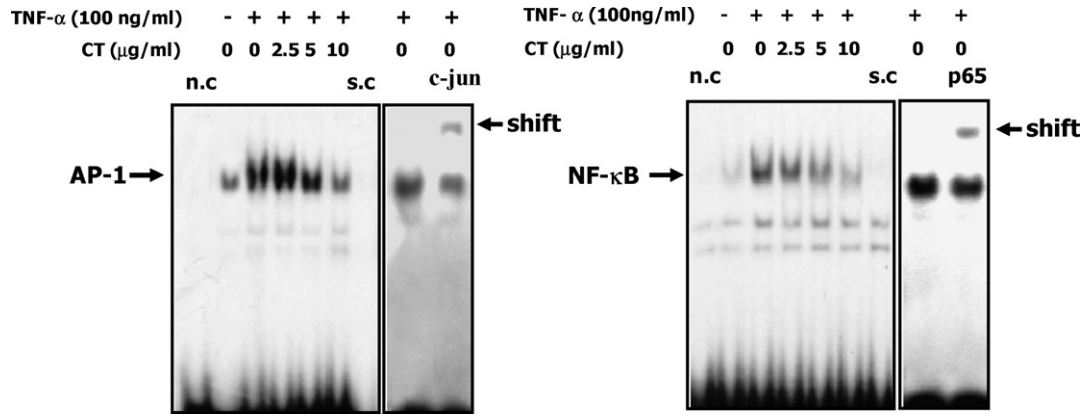


Fig. 5 – Electrophoretic mobility shift was performed with NF-κB and AP-1 specific probes. Protein samples were prepared from HASMC after treatment of several doses of CT-treated or non-treated with 100 ng/ml of TNF-α. NF-κB and AP-1 nuclear translocation was lowered when CT treated with a dose dependent manner. The supershift assay was performed using 10 X phosphor-p65 and 10 X phosphor-c-jun antibodies. n.c: negative control, s.c: specific competeter.

of MMP-9) was used in this study and described in Fig. 4A. As shown in Fig. 4C, luciferase activity was increased up to nine-fold in HASMC cells that had been treated with TNF-α compared with untreated cells. On the other hand, luciferase activity of cells treated with CT (10 μg/ml) in the presence of TNF-α was reduced by 2- or 2.5-fold, respectively, compared with that of TNF-α stimulated. Because MMP-9 gene expression in HASMC was regulated by the transcription factors such as NF-κB and AP-1 [34], in the following, it was determined whether NF-κB and AP-1 activated by TNF-α were inhibited by CT.

3.4. *CT Inhibits NF-κB and AP-1 activation induced by TNF-α*

TNF-α is a potent activator of NF-κB and AP-1 and presumptive binding site of these transcription factors were located in MMP-9 promoter region. To further confirm that CT is directly involved in the inhibition of NF-κB and AP-1 mediated transcriptional activation of MMP-9, we examined the inhibitory effect of CT on the binding of AP-1 and NF-κB isolated from TNF-α-treated HASMC to oligonucleotides that contain the sequence for the AP-1 and NF-κB binding sites using EMSA. The results of EMSA showed that CT suppresses the NF-κB and AP-1 induced by TNF-α (Fig. 5). As shown in Fig. 5, when the [γ-^{32}P]-labeled NF-κB and AP-1 oligonucleotide were incubated with nuclear extract from TNF-α-treated HASMC, the signal were retarded. The nuclear extract from CT-treated HASMC showed weak retarded signal dose dependently. Therefore, it is supported that MMP-9 transcription was affected by down-regulation of these nucleus factors.

3.5. *CT inhibits ERK1/2, p38 and JNK phosphorylation*

As reported previously, MMP-9 expression is dependent to NF-κB and AP-1 nuclear translocations, Furthermore, ERK1/2 signaling pathway had important role to NF-κB and AP-1

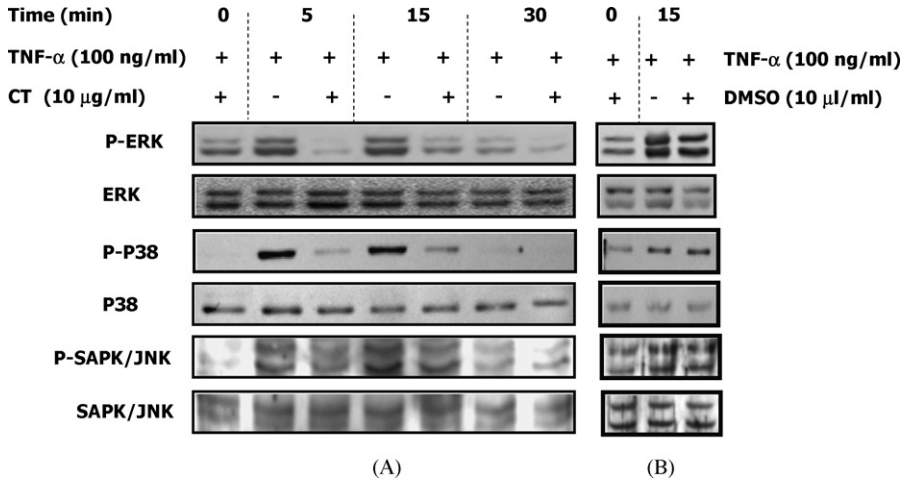


Fig. 6 – CT suppresses MAP kinase signals containing the ERK 1/2, p38, JNK. (A) In Western blotting, the phospho-ERK 1/2, phosho-p38 and phospho-JNK level was measured in early time period (5, 15 and 30 min) at 10 μg/ml CT-treated or non-treated with 100 ng/ml of TNF-α. CT significantly suppressed the phosphorylated ERK 1/2, phosphorylated p38 and also the phosphorylated JNK levels. (B) Vehicle solution DMSO (10 μl/ml) was used as a control reagent and repeated the Western blotting after 15 min stimulation.

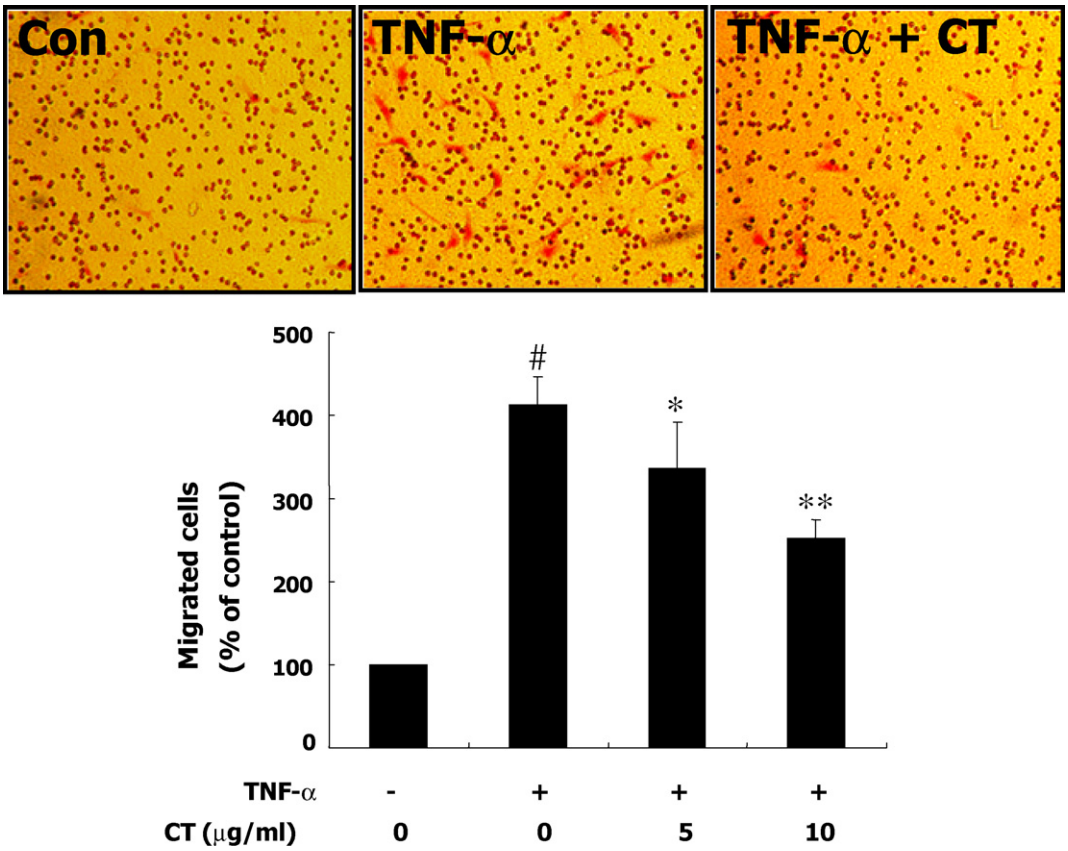


Fig. 7 – In matrigel tanswell system, the TNF-α stimulated HASMC was migrated highly through matrigel, but the CT suppressed the migration as normal level. Transwells were coated with matrigel. HASMC and media treated with various concentrations of TNF-α and CT were added to matrigel-coated transwell. The downward of 8 μm pore membrane of transwell that contain HASMC was stained with hematoxylin and eosin Y and the migrated cell number were counted as an average of three independent experiments. The data represent the mean ± S.E. of at least three independent experiments. Results were statistically significant ($^{\#}p < 0.05$ compared with control, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with TNF-α only stimulation, respectively, using Student's t-test).

dependent promoter activity [34]. The phosphorylated ERK1/2 was up-regulated when treating TNF-α and then the nuclear NF-κB and AP-1 were increased [35]. To examine the effect of CT on the activation of Erk by TNF-α, we treated cells with CT for 2 h, and stimulated with TNF-α by the time course (0, 5, 15, and 30 min). Then, we prepared whole cell extract, and performed Western blot analysis. In Fig. 6, the stimulated p-ERK level was down-regulated when CT treated (10 μg/ml). As previously reported, the phospho-p38 was up-regulated when TNF-α treated, and p-p38 level also affected the MMP-9 productivity [9]. The total cell lysates of the 5 and 15 min after of TNF-α-treated HASMC showed induced levels of phospho-p38 and CT treated cells significantly suppressed it. In the next time, we investigated the p-JNK induction in TNF-α-treated HASMC in the same conditions, because it is reported that TNF-α-treated VSMC also activated JNK signaling [9]. The performed Western blotting using anti-phospho-specific-JNK antibody showed CT suppressed TNF-α induced phospho-JNK. With these results, it is postulated that CT is a potent inhibitory compound of MAP kinase and this pathway is important to produce MMP-9.

3.6. *CT inhibits the TNF-α induced HASMC invasion*

We previously showed that CT from *S. miltiorrhiza* BUNGE suppresses NF-κB and AP-1 mediated MMP-9 expression in TNF-α-induced HASMC cells. Because the up-regulation of MMP-9 expression might be expected to contribute to an invasive phenotype, we examined whether the invasiveness of TNF-α stimulated HASMC was decreased by CT (10 μg/ml). As shown in Fig. 7, the invasiveness of TNF-α induced HASMC was increased when compared with the invasiveness of TNF-α untreated HASMC. As evidenced by a Matrigel invasion assay, CT blocked the invasiveness of TNF-α induced HASMC. From these results, we conclude that with respect to HASMC, CT induces a decrease in the potential for invasion.

4. Discussion

Proliferation and migration of VSMC play an important role in the pathogenesis of atherosclerosis and restenosis after vascular injury [36]. On injury, VSMC migrate from the tunica

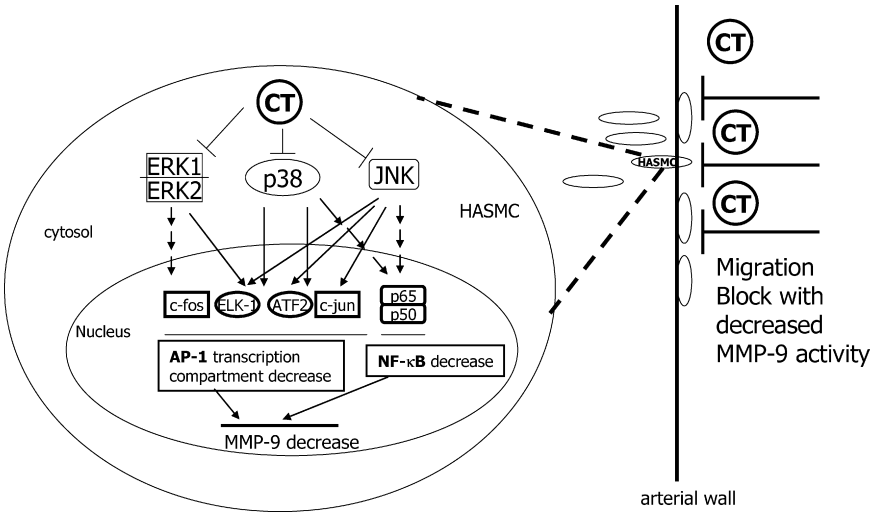


Fig. 8 – The schematic diagram of mechanisms of anti-atherosclerosis activity of CT. When arterial wall was injured, HASMC was activated by the TNF-α. The up-regulation of MMP-9 production and cell migration was inhibited by CT. In the figure, CT blocked the p-JNK, p-p38, and p-ERK and the down-stream transcription factors, AP-1 and NF-κB were downed. By the way of inhibiting these signaling, the migration via MMP-9 gelatinolytic activity was blocked in the arterial wall.

media to the intima, leading to neointima formation [37]. As VSMC in the media are entirely surrounded by basal lamina and ECM, VSMC migration requires breakdown of ECM [38,39]. Among these extracellular proteinases, the MMPs have been shown to play an essential role [3,38,40].

Several natural products have been used for atherosclerosis [25], especially baicalein [41] and green tea catechins [42], which inhibit proliferation and migration of VSMC, respectively. In addition, anti-oxidant fraction of *S. miltiorrhiza* inhibited intimal hyperplasia after balloon injury [28]. From these circumstances, we have investigated the effects of CT, the one of major compounds purified from *S. miltiorrhiza*, on migration and the MMP-9 activity of HASMC. Because MMP-2 activity was not changed or minimally affected by TNF-α or CT, we proposed pro-MMP-2 was constantly expressed in our conditions. Therefore, in this study, we focused on MMP-9 and its inhibition mechanisms by CT. The migration of HASMC cells was significantly increased by TNF-α treatment in good concordance with previous report. However, CT inhibited migration of the TNF-α-treated HASMC. The TNF-α induced MMP-9 activity was suppressed in the serum free culture with CT. Previous studies in our laboratory, as well as others, have demonstrated that TNF-α induced transcriptional MMP-9 promoter activity in VSMC through AP-1 and NF-κB [30,34,43]. This result was confirmed by transfecting the pGL2-WT-MMP-9 pro plasmid, the promoter region of which contains binding sites for the NF-κB and AP-1 transcription factors. And then, we have further investigated whether the decreased binding activities of NF-κB and AP-1 was resulted from the negative effect of CT on NF-κB and AP-1 levels. By using consensus AP-1 and NF-κB probes, a marked decrease in both AP-1 and NF-κB binding activities in response to TNF-α in HASMC following treatment with CT was observed.

Recent studies have demonstrated that TNF-α induced MMP-9 expression in VSMC is mediated by increased activities of NF-κB and AP-1 and involves the activation of the Ras/ERK1/2 pathway [30,33,34]. To explain this inhibition mechanism against TNF-α-induced MMP-9 production via nucleus NF-κB and AP-1 levels, we assessed the phosphorylation event of ERK1/2 and p38 in the TNF-α induced HASMC. By the Western blot analysis the phosphorylated ERK and p-38 was decreased when treating 10 μg/ml CT. At the same time, the other one of MAP kinase, JNK was stimulated in TNF-α induced VSMC, but not in PDGF-treated VSMC [9]. Therefore, we searched whether phospho-JNK level was involved in TNF-α-treated HASMC and CT could suppress it. In Western blotting, phosphorylated JNK was increased by TNF-α and suppressed by CT at 10 μg/ml. These results and other researcher's evidences could explain the CT's inhibitory effect of the MAP kinase activity results in decrease of MMP-9 secretion involving cardiovascular disease [9,34].

In the herbal medicine, the *S. miltiorrhiza* BUNGE is one of the main curing herbs for cardiovascular disease and many researchers attempt to purifying and analyzing the compounds from it. One of the purified compounds, CT, had been reported as inhibitor of NO generation and anti-angiogenesis material [29,44]. Therefore, we attempt to characterize CT's bioactivity for MMP-9 production and invasion in HASMC after TNF-α treating. CT's inhibition mechanism was elucidated as many ways of promoter assay, EMSA, and Western blotting analysis, so we could suggest that the transcriptional down-regulation of MMP-9 is probably involved in decreased nucleus proportion of NF-κB and AP-1 via MAP kinase including ERK1/2, p38 and JNK signaling pathways as shown in Fig. 8. Taken together, we suggest that the CT has potential anti-atherosclerotic capability of future therapeutic medicine.

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