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Inhibitory effects of caffeic acid phenethyl ester on cancer cell metastasis mediated by the down-regulation of matrix metalloproteinase expression in human HT1080 fibrosarcoma cells

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Abstract

Caffeic acid phenethyl ester (CAPE) derived from honeybee propolis has been used as a folk medicine. Recent study also revealed that CAPE has several biological activities including antioxidation, anti-inflammation and inhibition of tumor growth. The present study investigated the effect of CAPE on tumor invasion and metastasis by determining the regulation of matrix metalloproteinases (MMPs). Matrix metalloproteinases, which are zinc-dependent proteolytic enzymes, play a pivotal role in tumor metastasis by cleavage of extracellular matrix (ECM) as well as nonmatrix substrates. On this line, we examined the influence of CAPE on the gene expression of MMPs (MMP-2, MMP-9, MT1-MMP), tissue inhibitor of metalloproteinase-2 (TIMP-2) and in vitro invasiveness of human fibrosarcoma cells. Dose-dependent decreases in MMP and TIMP-2 mRNA levels were observed in CAPE-treated HT1080 human fibrosarcoma cells as detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Gelatin zymography analysis also exhibited a significant down-regulation of MMP-2 and MMP-9 expression in HT1080 cells treated with CAPE compared to controls. In addition, CAPE inhibited the activated MMP-2 activity as well as invasion, motility, cell migration and colony formation of tumor cells. These data therefore provide direct evidence for the role of CAPE as a potent antimetastatic agent, which can markedly inhibit the metastatic and invasive capacity of malignant cells.

Keywords: Caffeic acid phenethyl ester (CAPE); Extracellular matrix (ECM); Matrix metalloproteinase-2 (MMP-2); Matrix metalloproteinase-9 (MMP-9); Metastasis

1. Introduction

Propolis is extracted from the bark of conifer trees and carried by honeybees to their hives. Along with a variety of its beneficial effects, it has been a popular folk medicine through the ages. Caffeic acid phenethyl ester (CAPE) (Fig. 1) [1] extracted from honeybee propolis is a biologically active ingredient of propolis with several interesting biological properties. Besides their well-known antioxidant activity [2,3], CAPE inhibits certain enzyme activities such as lipoxygenases, cyclooxygenase, glutathione *S*-transferase and xanthine oxidase [4–7]. Caffeic acid phenethyl ester has also been reported to have antitumor activity [8,9], anti-inflammatory properties [5,10], apoptosis inducible functions [11], inhibitory effects of HIV replication [12,13] and antimetastatic activity in CT26 colon adenocarcinoma cells of BALB/c origin [14]. CAPE-treated CT26 cells showed not only inhibited cell invasion but also decreased expression of matrix metalloproteinases (MMPs). Chung et al. [15] have also shown that CAPE selectively inhibited MMP-2 and MMP-9, but not MMP-1, MMP-3, MMP-7 or cathepsin K, and significantly reduced the liver metastasis.

Matrix metalloproteinases are a multigene family of zinc-dependent endopeptidases capable of degrading essentially all extracellular matrix (ECM) components, and MMPs have been considered to play an important role in matrix degradation for tumor growth, invasion and tumor-induced

Abbreviations: APMA, *p*-aminophenyl mercuric acetate; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; MMPs, matrix metalloproteinases; MT1-MMP, membrane-type metalloproteinase; RT-PCR, reverse transcriptase-polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinase.

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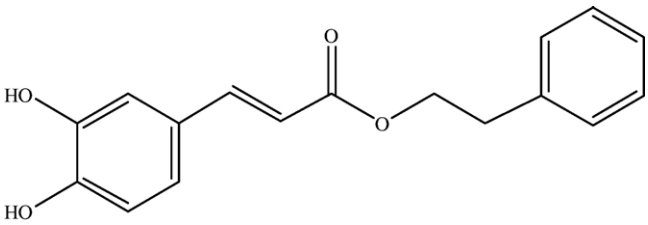

Fig. 1. Chemical structure of CAPE.

angiogenesis [16]. Among MMPs, MMP-2 and MMP-9 are considered to play critical roles during tumor invasion and metastasis [17]. The MMP activity is also finely regulated on many levels including transcriptional control, proenzyme activation, and inhibition of activated MMPs by nonspecific inhibitors such as α2-macroglobulin [18], or more specific endogenous inhibitors such as tissue inhibitors of metalloproteinase (TIMPs) [19]. TIMPs bind to the active site of MMPs and block access of MMPs to their substrates. As suggested, MMPs are also regulated by endogenous modulators with various mechanisms [20]. In particular, MMP-2 is secreted from cells as a zymogen (pro-MMP-2) and is activated post-translationally by a trans-membrane MMP designated as membrane type 1 MMP (MT1-MMP) [21]. The activation of pro-MMP-2 is regulated by a complex mechanism involving the formation of a trimolecular complex with MT1-MMP and TIMP-2 [21,22]. TIMP-2 bridges the interaction between the MMP-2 zymogen and MT1-MMP via N-terminal binding to the active site of MT1-MMP with the concomitant C-terminal binding to the pro-MMP-2 hemopexin domain [22,23]. In addition, Coussens et al. [24] have shown that MMP-9 as well as MMP-2 is functionally involved in progression to invasive cancer and regulation of oncogene.

In this study, we further investigate the influence of CAPE on the gene expression of MMPs (MMP-2, MMP-9, MT1-MMP) and TIMP-2 and in vitro invasiveness of human fibrosarcoma cells.

2. Materials and methods

2.1. Materials

All media for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). *p*-Aminophenyl mercuric acetate (APMA), CAPE, human type I collagen, 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly, bovine serum albumin and gelatin were purchased from Sigma (St. Louis, MO, USA). Pro-MMP-2 was purchased from Bristol-Myers Squibb (Princeton, NJ). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA). All other chemicals used were of reagent grade.

2.2. Cell culture

HT1080 human fibrosarcoma cells were grown in DMEM supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin and 10% heat-inactivated fetal bovine serum. Cultures were maintained at 37 °C in a 5% CO_2/95% air atmosphere.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by the use of TRI reagent (Sigma) and reverse-transcribed at 42 °C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 U/μl of recombinant RNasin ribonuclease inhibitor, 15 U/μg of AMV reverse transcriptase and 0.5 μg of oligo$(dT)_{15}$ primer. For the determination of target genes, the following primers were designed using primer3: MMP-2 (forward, +1689–1713; reverse, +2055–2079), MMP-9 (forward, +1238–1260; reverse, +1613–1637), TIMP-1 (forward, +233–252; reverse, +613–632), TIMP-2 (forward, +450–473; reverse, +828–850), MT1-MMP (forward, +1320–1342; reverse, +1813–1836). Sequences of the primer pairs were used in the PCR reaction (Table 1). The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA, USA), 2 μl of the reverse transcriptase reaction and 20 pmol of primer pairs in a total volume of 50 μl. Thermocycling was performed according to the following profile: 94 °C for 30 s, followed by 94 °C for 30 s, 55 °C for 30 s and 72 °C 30 s, repeated 25 times. Amplification was linear within the range of 20–30 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CT, USA).

2.4. Zymography

MMP-2 and MMP-9 enzymatic activities were assayed by gelatin zymography [26]. All experiments, including zymography, were performed in the absence of serum. Samples were electrophoresed on a gelatin containing 10% SDS-polyacrylamide gel. After electrophoresis, the gel was washed twice with washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a

Table 1
Sequences of the primer pairs employed in the RT-PCR reactions

Studied gene	Sequences of the primer pairs (5′→3′)	Product size (bp)
MMP-2	Forward: AGATCTGCAAACAGGACATTGTATT	400
	Reverse: TTCTTCTTCACCTCATTGTATCTCC	
MMP-9	Forward: CTGGGCTTAGATCATTCCTCAGT	400
	Reverse: AGTACTTCCCATCCTTGAACAAATA	
TIMP-2	Forward: GTCAGTGAGAAGGAAGTGGACTCT	401
	Reverse: ATGTTCTTCTCTGTGACCCAGTC	
MT1-MMP	Forward: GGGCCTGCCTGCGTCCATCAACA	400
	Reverse: GCCGCCCTCCTCGTCCACCTCAAT	
β-Actin [25]	Forward: AGCACAATGAAGATCAAGAT	188
	Reverse: TGTAACGCAACTAAGTCATA	

brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) at 37 °C. After incubation, the gel was stained and destained. A clear zone of gelatin digestion was represented with the MMP activity.

2.5. MMP-2 fluorometric assay

Fluorometric assay for the proteolytic activity of MMP-2 was performed using an MMP-2 substrate (7-methoxy-coumarin-4-acetyl-Pro-Leu-Gly). Briefly, Pro-MMP-2 was activated by incubation at 37 °C for 2 h in 0.5 mM APMA in a TNBC buffer containing 20 mM Tris–HCl, 5 mM $CaCl_2$, 0.15 M NaCl, pH 7.5. Activated MMP-2 was reacted with fluorescence substrate and CAPE at 37 °C for 3 h. Thereafter, fluorescence was measured using a fluorometer with excitation at 360 nm and emission at 420 nm.

2.6. Cell invasion and motility assay

Cells (5×10^4 cells/chamber) were used for each invasion assay. The lower and upper parts of the Transwell insert (Corning Costar, Corning, NY) were coated with 10 μl of type I collagen (0.5 mg/ml) and 20 μl of 1:2 mixture of Matrigel/DMEM, respectively. Cells were plated on Matrigel-coated Transwell inserts. The medium of the lower chambers contained 0.1 mg/ml bovine serum albumin. The inserts were incubated for 18 h at 37 °C. The cells that had invaded the lower surface of the membrane were fixed with methanol, stained with hematoxylin and eosin, and photographed.

To determine the effect of CAPE on cell motility, cells were seeded onto Transwell inserts on membrane filters coated with 10 μl of type I collagen (0.5 mg/ml) at the bottom of the membrane. Motility in the absence or presence of CAPE was measured as described in the invasion assay. In addition, cell motilities were measured using a wound-healing method and colony dispersion assay. Briefly, for a wound-healing assay, cells were grown almost confluently, and a wound was created with the blunt end of a yellow tip. This was documented through time-lapse photography. For a colony dispersion assay, 1 ± 10^4 cells in 20 μl were seeded in growth medium in the middle of a 24-well plate. At 6 h after plating, the medium was removed, and the cells were covered with DMEM with 1% fetal bovine serum for 2 h and then exposed to CAPE for an additional 48–72 h. After washing with phosphate-buffered saline, the cells were fixed with 4% formaldehyde and stained with hematoxylin and eosin. To quantify the expansion of the outgrowth, migratory monolayers were documented through digital photography (Olympus, Japan) with light.

2.7. Statistical analysis

Statistical analyses were performed using Student's *t* test and one-way ANOVA. $P<.05$ and $P<.01$ were considered

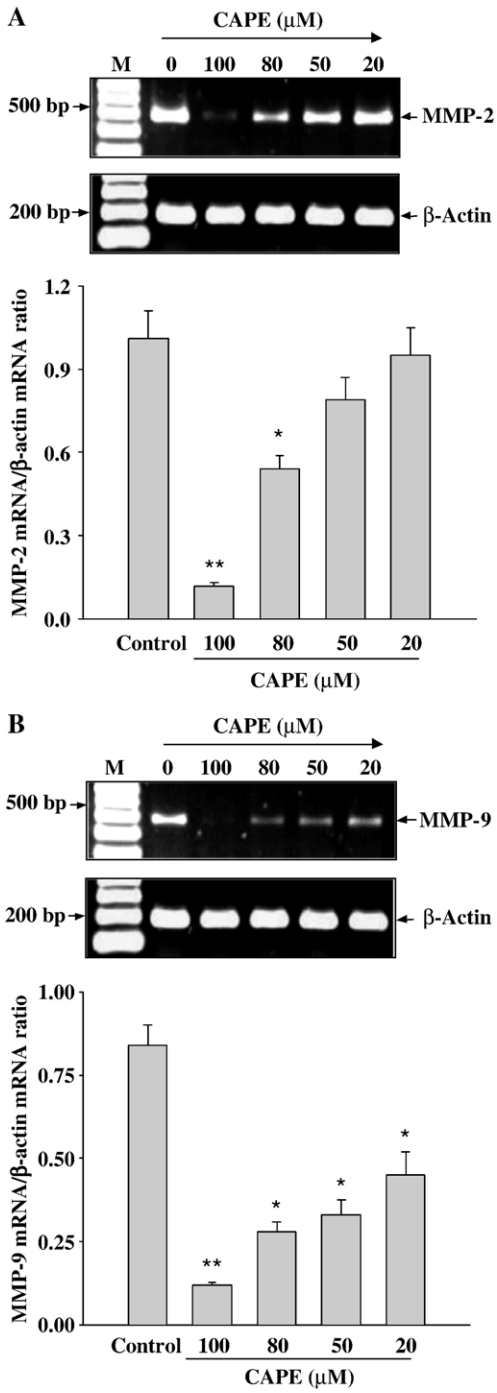

Fig. 2. Dose-dependent down-regulation of the (A) MMP-2 and (B) MMP-9 mRNA expression in HT1080 cells by CAPE using RT-PCR analysis. HT1080 cells were treated with various concentrations of CAPE for 24 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging (upper panel), followed by densitometric measurements (lower panel). The predicted sizes of RT-PCR products for MMP-2, MMP-9 and β-actin are 400, 401 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder). Data shown are the means±S.D. of three determinations ($n=3$). Treatment groups were significantly different from the untreated control group (*$P<.05$, **$P<.01$) by analysis of Student's *t*-test.

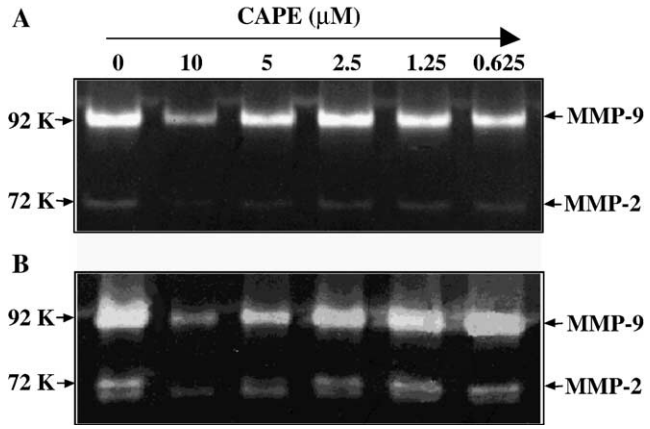

Fig. 3. Effects of CAPE on the activities of MMP-2 and MMP-9. HT1080 cells were treated with various concentrations of CAPE. After (A) 48 h or (B) 72 h, conditioned media were collected and analyzed by gelatin zymography. Data shown are representatives of three independent experiments.

statistically significant. All statistical analyses were performed using SPSS 10.0 software.

3. Results

3.1. CAPE inhibits the mRNA and protein expression of MMP-2 and MMP-9 in HT1080 cells

To investigate whether CAPE can inhibit the expression of MMP-2 and MMP-9, HT1080 human fibrosarcoma cells were treated with increasing concentration of CAPE (0, 20, 50, 80 and 100 μM), and the mRNA levels of MMP-2 and

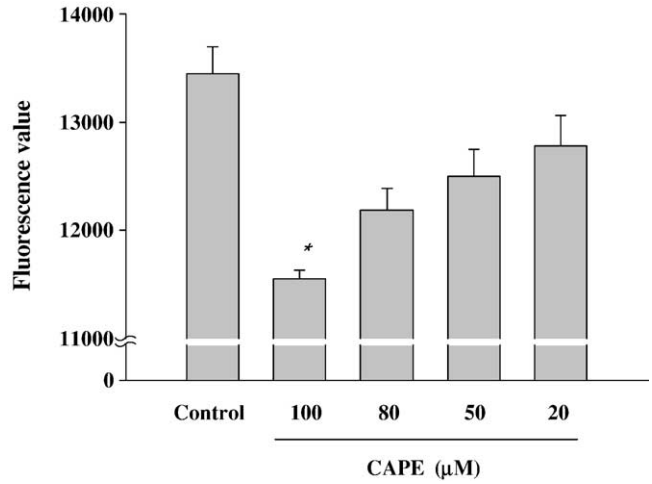

Fig. 4. Inhibitory effect of CAPE on the activities of MMP-2 in HT1080 cells. Fluorometric assay for the proteolytic activity of MMP-2 was performed using 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly. Pro-MMP-2 was activated by incubation with 0.5 mM APMA at 37 °C for 2 h. Activated MMP-2 was reacted with fluorescence substrate and CAPE at 37 °C for 3 h. Fluorescence was measured with excitation at 360 nm and emission at 420 nm. Data shown are the means±S.D. of three determinations ($n=4$). Sample groups were significantly different from the control group (*$P<.05$) by analysis of Student's t-test.

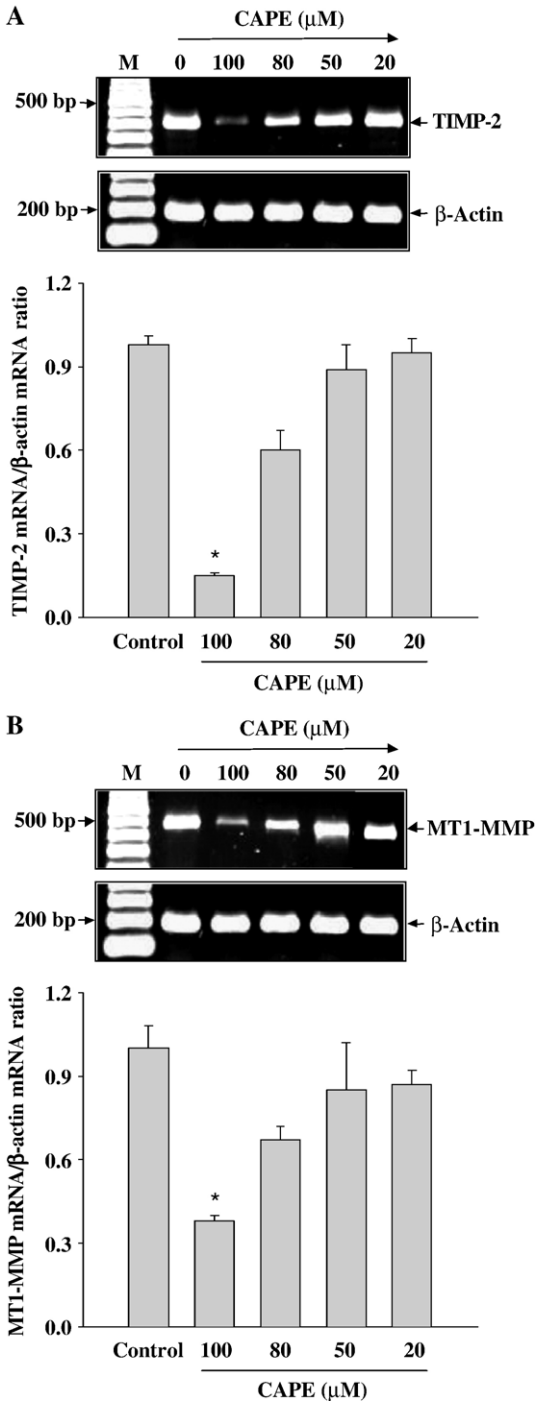

Fig. 5. Dose-dependent down-regulation of the (A) TIMP-2 (B) MT1-MMP mRNA expression in HT1080 cells by CAPE using RT-PCR analysis. HT1080 cells were treated with various concentrations of CAPE for 24 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging (upper panel), followed by densitometric measurements (lower panel). The predicted sizes of RT-PCR products for TIMP-2, MT1-MMP and β-actin are 401, 400 and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder). Data shown are the means±S.D. of three determinations ($n=3$). Treatment groups were significantly different from the untreated control group (*$P<.05$) by analysis of Student's t-test.

MMP-9 were determined by semiquantitative RT-PCR technique. As shown in Fig. 2A and B, mRNA transcripts for MMP-2 and MMP-9 were clearly inhibited in CAPE-treated cells. Down-regulation of MMP-2 and MMP-9 expression by treatment of CAPE was observed in a dose-dependent manner. In addition, gelatin zymographic analysis revealed that MMP-2 and MMP-9 proteins were constitutively expressed in untreated HT1080 cells, but protein expressions were markedly down-regulated and were dose-dependent in the cells treated with CAPE for 48 h (Fig. 3A) and 72 h (Fig. 3B).

3.2. Effects of CAPE on MMP-2 activity

To investigate whether CAPE can inhibit activated MMP-2 activity, pro-MMP-2 was subjected to activation studies using the organomercuric compound, APMA, which activates MMP-2 by inducing autocatalytic cleavage of the pro-enzyme. Assay mixtures were reacted with CAPE, a 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly substrate, and activated MMP-2 for 3 h. As illustrated in Fig. 4, CAPE significantly inhibited the MMP-2 activity in a dose-dependent manner.

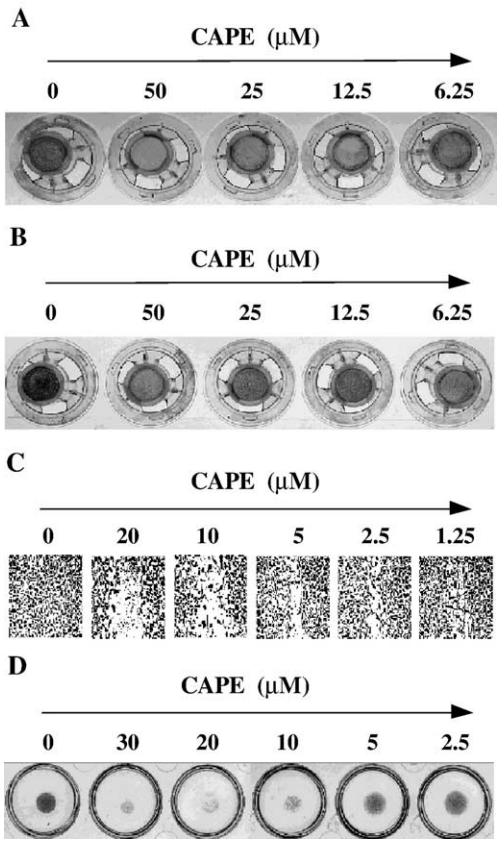

Fig. 6. Effect of CAPE on invasion, motility, wound healing and colony dispersion assay. (A) HT1080 cells were treated with 0, 6.25, 12.5, 25 or 50 μM CAPE for 18 h using Matrigel-coated Transwell inserts. (B) The same method used in (A) except the upper part of the Transwell inserts was coated with Matrigel. (C) Confluent cultures of HT1080 cells were wounded with a tip. The cells were incubated with increasing concentrations of CAPE for 16 h, fixed and stained. (D) HT1080 cell migration was determined after 3 days of incubation by various concentrations of CAPE shown in a colony dispersion assay. Data shown are representatives of three independent experiments.

3.3. CAPE down-regulates TIMP-2 and MT1-MMP genes in HT1080 cells

To further explore the modulation of activation of pro-MMPs mediated by CAPE, TIMP-2 and MT1-MMP gene expressions were investigated. As shown in Fig. 5A, CAPE decreased TIMP-2 mRNA level. MT1-MMP mRNA levels were also suppressed by the treatment of CAPE, and dose-dependent down-regulation was observed at 24 h (Fig. 5B), demonstrating that CAPE suppresses the expression of TIMP-2 and MT1-MMP mRNA levels.

3.4. Effects of CAPE on invasion and migration activity

HT1080 cells have an ability to invade through Matrigel. Treatment of CAPE for 16 h exhibited a significant inhibition of cell invasion in a dose-dependent manner (Fig. 6A). Cell motility through Transwell (Fig. 6B) and spreading onto plastic ware (Fig. 6C) were also inhibited by treatment of CAPE. In colony dispersion assay, cells were plated onto the middle of a 24-well plate with a high density and migrated as an outgrowth. As shown in Fig. 6D, HT1080 cells treated by CAPE inhibited the migration ability in a dose-dependent manner.

4. Discussion

Cancer cell–matrix interaction is a critical step that promotes cell migration, proliferation and degradation [27,28]. Proteolytic degradation of ECM is a critical event during tumor invasion and metastasis. Although breakdown of the basement membrane is achieved by several MMPs, MMP-2 and MMP-9 appear to be most important for basement membrane type IV collagen degradation [27–29]. Recently, CAPE has seen considerable interest in antimetastatic activity in mouse colon carcinoma cells (CT 26) [14] and inhibitory effect of invasion and metastasis in human hepatocellular carcinoma cell line (HepG2) [15]. Therefore, it is important to characterize the effect of CAPE on the expression of MMPs and TIMPs in greater detail. HT1080 cells, a human fibrosarcoma cell line, have been used extensively as a model to study the migration and invasion by tumor cells. Similar to various malignant tumors including human colon cells, HT1080 cells express MMP-2 and MMP-9 at a high level [30].

In the present study, we explored whether CAPE modulates cancer invasion and metastasis in cultured HT-1080 cells using MMP expressions as representative biomarkers. As a result, CAPE significantly suppressed the gene expression of MMPs (MMP-2, MMP-9, MT1-MMP) and TIMP-2 in HT1080 human fibrosarcoma cells, and also nontoxic ranges of CAPE markedly decreased MMP activity, cell migration, motility and invasiveness.

Matrix metalloproteinases mediate invasion and metastasis through degradation of the ECM and basement membrane, allowing tumor cells to invade surrounding tissues and enter the blood stream to travel to distant sites [31]. Especially, MMP-2 and MMP-9 are considered to be particularly important targets for development of anticancer drugs because both enzymes degrade type IV collagen, a major component of the basement membrane, and also the expression of both enzymes correlates with an aggressive, advanced, invasive or metastatic tumor phenotype [32]. Furthermore, knockout mice for either gelatinase have reduced tumor burden, decreased metastasis, as well as reduced tumor angiogenesis without developmental abnormalities [17,33]. On this line, CAPE-mediated suppression of MMP-2 and MMP-9 gene expression (Fig. 2A and B) suggests that CAPE might inhibit the proteinase expression giving reasonable explanation for the inhibition of cancer cell invasion. In addition, gelatin zymographic analysis showed that down-regulation of the MMP-2 and MMP-9 genes by CAPE is well correlated with a significant and dose-dependent suppression of MMP-2 and MMP-9 protein in HT1080 cells (Fig. 3). Moreover, CAPE inhibited the activated MMP-2 activity significantly in a dose-dependent manner (Fig. 4).

In addition, TIMPs play an important role in regulating the activity of the secreted metalloproteases. Among them, TIMP-2, a 21-kDa nonglycosylated protein, selectively forms a complex with the 72-kDa procollagenase (proMMP-2) at the active site. In the present study, CAPE suppressed TIMP-2 mRNA levels (Fig. 5A) as well as MMP-2 mRNA. Although TIMP-2 is considered to be an inhibitor of MMP-2, expression of this inhibitor is differentially regulated in vivo as well as in a cell culture system [34]. These effects on MMPs and TIMPs are not just confined to CAPE. Genistein and selenite decrease MMP-2 and MMP-9 mRNA levels, whereas these compounds increase TIMP-1 mRNA levels but decrease TIMP-2 mRNA in MDA-MB-231, MCF-7 and HT1080 fibrosarcoma cells [35,36]. Ursolic acid (UA) decreases MMP-9, but MMP-2 and membrane-type MMP were constantly expressed, and TIMP-1 and TIMP-2 were not changed either after 3 and 6 days of treatment with UA in HT1080 cells [37]. 1α,25-Dihydroxyvitamin D_3 and its analogues also down-regulate MMP-9 and uPA, whereas it up-regulates TIMP-1 and uPAI-1 levels in MDA-MB-231 cells [38]. Silibinin, a flavonoid antioxidant, markedly decreased MMP-2 and uPA level and increased TIMP-2 protein level without affecting the mRNA level and TIMP-1 protein or mRNA levels [39].

Pro-MMP-2 can be activated by several mechanisms depending on stimulators and cell types. Initially, pro-MMP-2 can be activated by the action of highly expressed MT1-MMP and the adequate expression of TIMP-2 [40–42]. In this situation, the balance between MT1-MMP and TIMP-2 is important. At low concentration, TIMP-2 binds to the catalytic site of some activated MT1-MMP molecules, generating receptors for pro-MMP-2, thereby promoting MMP-2 activation.

In this study, CAPE-down-regulated MMP-2 gene expression is, at least in part, mediated at the level of transcription regardless of TIMP-2 gene expression. Therefore, CAPE-treated HT1080 cells, at least in part, decreased pro-MMP-2 activation through the suppression of MT1-MMP (Fig. 5B) and not adequate expression of TIMP-2. In accordance with these results, CAPE inhibited not only an in vitro invasion and motility in the Matrigel model but also migration and colony dispersion of HT1080 cells (Fig. 6).

In conclusion, we here report that CAPE inhibited invasion- or metastasis-associated protease activities. The inhibition was also related to the suppression of transcriptional level; however, its mechanism of action still remains elusive. In addition, CAPE inhibits degradation and cellular invasion of ECM and basement membrane. This study provides an additional activity of antimetastatic potential beyond antitumor activity mediated by CAPE.

Acknowledgments

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