

行政院國家科學委員會專題研究計畫 成果報告

醣基在類黃酮化合物體內與體外抗直腸腫瘤活性之角色與
機轉探討:訊息傳導, 端粒酵素活性與細胞凋亡之相關性

計畫類別：個別型計畫

計畫編號：NSC94-2320-B-038-049-

執行期間：94 年 08 月 01 日至 95 年 07 月 31 日

執行單位：臺北醫學大學生藥學研究所

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 95 年 10 月 24 日

執行期間：2005.08.01 至 2006.07.31

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中 華 民 國 95 年 10 月 23 日

中文摘要

本計劃探討類黃酮化合物myricetin (ME) 與其醣化物myricitrin (MI) 對直腸癌細胞轉移與MMPs活性之影響。結果顯示ME 而非MI有效抑制直腸癌細胞COLO 320HSR, COLO 320DM, HT 29 and COLO 205-X 之轉移其 IC_{50} 分別為 11.18, 11.56, 13.25 and 23.51 M。我們更進一步分析其抑制機轉，結果顯示ME之抑制活性與抑制MMP2 基因表現與酵素活性，抑制PKC活化、ERKs磷酸化與c-Jun蛋白表現被證實在ME處理細胞中 (This paper has been accepted by Molecular Cancer Therapy (2005)).

Abstract

Colorectal carcinoma is one of the leading causes of human mortality due to its high metastatic ability via activation of matrix metalloproteinases (MMPs). Therefore, agents with ability to inhibit MMPs activity possess potential in treatment of colorectal carcinoma. In the present study, among thirty-six flavonoids examined, myricetin (ME) was found to be the most potent inhibitor of MMP-2 enzyme activity in COLO 205 cells with an IC_{50} value of 7.82 M. ME inhibition of MMP-2 enzyme activity was also found in human colorectal carcinoma cells COLO 320HSR, COLO 320DM, HT 29 and COLO 205-X with IC_{50} values of 11.18, 11.56, 13.25 and 23.51 M, respectively. ME inhibition of MMP-2 enzyme activity was due to directly suppress MMP-2 enzyme activity with reduced MMP-2 protein expression. In contrast, no inhibitory effect of MMP-2 protein expression or enzyme activity was observed in myricitrin (MI; ME-3-rhamnoside)-treated cells. In the TPA stimulation condition, an increase in MMP-2 protein expression and enzyme activity was observed in according with inducing PKC protein translocation, ERK1/2 protein phosphorylation, and c-Jun protein expression in COLO 205 cells. ERKs inhibitor (PD98059) and PKC inhibitors (GF and H7), but not p38 inhibitor (SB203580) or JNK inhibitor (SP600125), significantly inhibited TPA-induced MMP-2 protein expression with reduced ERKs phosphorylation and c-Jun protein expression. Addition of ME but not MI suppressed TPA-induced MMP-2 protein expression with blocking the TPA-induced events including translocation of PKC from cytosol to membrane, phosphorylation of ERK1/2 protein, and induction of c-Jun protein expression in COLO 205 cells. Addition of PD98059 or GF significantly enhances the inhibitory effect of ME on MMP-2 enzyme activity induced by TPA. Furthermore, ME, but not MI, suppressed TPA-induced invasion of COLO205 cells in in vitro

invasion assay using EHS matrigel-coated transwells. Results of the present study indicated that ME significantly blocked both endogenous and TPA-induced MMP-2 enzyme activity via inhibition of its protein expression and enzyme activity. The blockade involved suppression of PKC translocation, ERKs phosphorylation, and c-Jun protein expression.

Key words: flavonoids, myricetin, MMP-2, invasion, TPA, ERKs

Introduction

Extracellular matrix (ECM) is a complex structure in supporting cells in mammalian tissues, and there are several biomolecules in ECM including collagen and casein in maintaining the three-dimensional structure of the body (1). During carcinogenesis, degradation of ECM has occurred in the process of metastasis of malignant tumors, and is involved in the migration and invasion of malignant tumor cells (2, 3). Matrix metalloproteinases (MMPs) are proteins for ECM breakdown, and activation of these protein activities has been detected in malignant tumors including colon carcinoma, lung cancer, and hepatoma (4, 5). Both MMP-2 and MMP-9 have been shown to participate in the tumor metastatic process and that the more malignant tumors are the more potent MMP-2 and MMP-9 activity is in stimulating metastasis of tumors (6, 7). Therefore, an agent capable of inhibiting activity of MMPs, especially MMP-2 and MMP-9, may potentially be an anti-tumor metastatic drug.

TPA, 12-O-tetradecanoylphorbol-13-acetate, a potent tumor promoter, induces a variety of cellular responses including differentiation, proliferation, and apoptosis. Protein kinase C (PKC) activation by TPA via translocation of PKC proteins from cytoplasm to membrane has been shown to be an essential step in TPA-induced tumor promotion (8). At least 5 isoforms of PKC including α , β , γ , δ , and ϵ have been isolated from different human tissues, and activation of PKC- α is involved in the formation of colorectal carcinoma. Following PKC activation, a series of downstream-genes such as mitogen activated protein kinases (MAPKs), c-Jun and c-Fos is stimulated. Our previous study indicated that induction of ERK1/2 phosphorylation, ornithine decarboxylase (ODC), c-Jun, and cyclooxygenase 2 (COX-2) proteins is involved in TPA-induced transformation in NIH3T3 cells. Zeliadt et al (9) indicated that TPA induced MMP-13 gene expression via an ERK-dependent pathway in mouse keratinocytes. In human cervical cancers, TPA induced MMP-9 activity via activation of PKC and JNK. Arnott et al (10) suggested TPA was able to up-regulate MMP-9 expression and was closely correlated with tumor development. However, the exact mechanism of TPA regulation of MMPs in colorectal carcinoma is still unclear.

Colorectal cancer is the third leading cause of cancer mortality in Western world, and increases strikingly. An early diagnosis and surgery to remove primary tumor have increased the survival rate of colorectal cancer patients. Several studies

indicated that metastasis is a major factor to cause death in colorectal cancer patients (11). Therefore, investigators keep continue to search effective agents to inhibit the metastasis of colorectal cancer.

Flavonoids are benzo- -pyronederivatives with different numbers of hydroxyl substitutions in the structures. Beneficial effects of flavonoids include anti-oxidant, anti-tumor and anti-inflammation (12,13). Results of structure activity relationship (SAR) studies have indicated that OH substitutions may affect the biological functions of flavonoids, and increasing OH substitution seem to enhance anti-oxidant activity of flavonoids. Our recent studies also demonstrated that flavonoids such as quercetin and wogonin induced apoptosis of tumor cells, and addition of glycoside attenuated the apoptosis-inducing activity of flavonoids (14,15). In addition, flavones with one or none OH substitution significantly inhibit epidermal growth factor (EGF)-induced proliferation in A431 (16). These data suggest that chemical structure especially OH substitution plays important roles in the biological activities of flavonoids. However, the SAR of flavonoids on inhibiting MMPs activity is still undelineated. In the present study, thirty-six structure-related flavonoids are used to investigate the inhibitory activities on MMPs activity in colorectal carcinoma cells. The results suggest that myricetin is the most potent inhibitor on MMP2 activity and TPA-induced cellular responses in COLO 205 cells. The inhibitory mechanism by myricetin via inhibiting MMP2 gene expression and enzyme activity, and SAR of flavonoids on MMP2 inhibition were elucidated.

RESULTS

ME suppression of MMP-2 enzyme activity in human colorectal carcinoma cells COLO 205 among thirty-six flavonoids

Thirty-six structure-related flavonoids including flavones, flavanones, and catechins were used to investigate the inhibitory effect on MMP-2 enzyme activity in human colorectal carcinoma cells COLO205. These compounds possess a basic benzo-pyrone in structure with different substitutions such as OH, OCH₃, and glycosides at different locations. Among them, flavones contain a double bond at C2-C3 of flavanones, and catechin possesses 5 OH substitutions at C3', 4', 3, 5, and 7, without an oxo group at C4 of flavanone. In order to investigate the inhibitory effect of tested compounds on matrix metalloproteinases (MMPs) activity, release of MMPs in the medium was performed by gelatin zymography. In COLO 205 cells, a detectable endogenous MMP-2, but not MMP-9, enzyme activity in the medium was examined in the absence of tested compounds. As elucidated in Table 1, myricetin (ME) at 200

M, exhibited 90 % inhibition of activity of MMP-2 enzyme released from COLO205 cells. Kaempferol (200 M) also showed 47 % inhibition of released MMP-2 enzyme activity in the medium. Other flavonoids examined in the present study exhibited less than 20 % inhibition of released MMP-2 enzyme activity.

Results of MTT assay showed that flavone, 3-OH flavone, 2'-OCH₃ flavone, 2'-OH flavanone, 4'-OH flavanone, 6-OH flavanone, quercetin, wogonin, luteolin, and baicalein, at 200 M, showed significant cytotoxicity (>50 %) in COLO205 cells. ME and kempferol at 200 M exhibited 20 and 30 % reduction in the viability of COLO 205 cells, respectively. Morphological observations and DNA integrity assay showed that ME did not change the morphology of COLO205 cells and no DNA laddering effect was detected in ME-treated COLO205 cells (Data not shown). These data suggest that ME is the most effective inhibitor among thirty-six flavonoids examined on activity of MMP2 released from COLO205 cells.

ME concentration-dependently inhibits MMP-2 enzyme activity in colorectal carcinoma cells COLO205, COLO320HSR, COLO320DM, HT-29, and COLO205-X

Previous data suggested that ME exhibited MMP-2 inhibitory effect without severe cytotoxicity in COLO205 cells. Therefore, several colorectal carcinoma cells

including COLO320HSR, COLO320DM, HT-29, and COLO205-X cells were used to investigate the MMP-2 inhibition by ME. As illustrated in Fig. 1A, structures of ME and its related glycosylated compound myricitrin (MI) were shown. MI contains a rhamnose at C3 of ME. ME, but not MI, dose-dependently inhibits MMP-2 enzyme activity released from COLO205, COLO320HSR, COLO320DM, HT-29, and COLO205-X cells (Fig 1B). The IC_{50} values of ME on MMP-2 activity in COLO205, COLO320HSR, COLO320DM, HT-29, and COLO205-X cells are 7.82, 11.18, 11.56, 13.25 and 23.51 μ M, respectively. MTT assay showed that ME only at 200 μ M exhibited slight but significant reduction on the viability of 205 cells. These data suggested that ME inhibition of MMP-2 enzyme activity was not due to its cytotoxic effect on cells.

ME inhibition of MMP-2 released from COLO205 via decreasing MMP-2 protein expression and inhibiting MMP-2 enzyme activity

Two possibilities might be involved in ME inhibition of activity of MMP-2 enzyme released from COLO205 cells; one is to block MMP-2 protein expression and the other is to directly inhibit MMP-2 enzyme. Results of Western blotting indicated that ME inhibited endogenous MMP-2 protein expression in COLO 205 cells without affecting that of MMP-1, MMP-3 and MMP-9 proteins. In contrast, MI did not affect any MMP protein expression in COLO205 cells (Fig 2A). Furthermore, the question that ME or MI might directly affect MMP-2 enzyme was examined by direct incubation of different concentrations of ME or MI with the medium taken from that culturing COLO205 at 37°C for 30 min, and MMP-2 activity was examined by gelatin zymography. It indicated that ME, but not MI, directly inhibited MMP-2 enzyme activity in vitro (Fig 2B). These data suggested ME inhibition of activity of MMP-2 enzyme released from COLO205 cells might be through both decreasing MMP-2 protein expression and/or suppressing MMP-2 enzyme activity.

ME directly inhibited MMP-2 activity using purified MMP-2 protein

In order to elucidate if ME directly blocked MMP-2 activity, a purified MMP-2 protein isolated from human fibroblasts was incubated with or without indicated doses (25, 50, 100, 200 μ M) of ME or MI at 37 °C for 30 min, and MMP-2 enzyme activity was analyzed by gelatin zymography. Results of Fig 2C & D indicated that purified MMP-2 protein was able to digest gelatin at 3 clear zones, and ME, but not MI, concentration-dependently inhibited MMP-2 enzyme activity as

indicated by a reduction in gelatin digestion in gel. Results from Western blot analysis using a specific MMP-2 antibody indicated that neither ME nor MI caused a decrease in MMP-2 protein level. These data provides direct evidence suggesting that ME directly inhibits MMP-2 activity.

TPA induction of MMP-2 activity in COLO 205 cells though PKC-translocation, ERK phosphorylation, and c-jun expression.

We further investigated if ME blocked 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-2 enzyme activity. TPA is a potent tumor promotor in carcinogenesis. As shown in Fig. 3A, TPA time-dependently induced both MMP-2 protein expression and enzyme activity released from COLO205 cells by Western blotting and gelatin zymography. An increase in c-Jun, but not MMP-1, MMP-3, and MMP-9, protein was observed in a time-dependent manner under TPA-treatment. Furthermore, PKC translocation from cytosol to membrane and activation of ERK1/2, but not p38 and JNKs, were detected in TPA-treated COLO205 cells by Western blotting. These data revealed events including induction of MMP-2 protein expression and enzyme activity, and increases in phosphorylated ERK1/2 protein and c-Jun protein expression with translocation of PKC from cytosol to membrane in TPA-treated COLO205 cells.

Phosphorylation of ERK1/2 proteins located at downstream of PKC translocation, in TPA-induced MMP-2 activity

In order to elucidate if phosphorylation of ERK1/2, translocation of PKC from cytosol to membrane and induction of c-Jun protein are essential events in TPA-induced MMP-2 enzyme activity, pharmacological studies using specific inhibitors including PD98059 (PD, MEK inhibitor), SB203580 (SB, p38 inhibitor), SP600120 (SP, JNK inhibitor), H-7 (PKA and PKC inhibitor), GF-109203X (GF, specific PKC inhibitor) were performed. The results indicated that TPA treatment induced significant morphological changes in COLO205 cells by microscopic observation with (lower pane) or without (upper panel) Giemsa staining (Fig. 4). Addition of PD, H-7, and GF, but not SB and SP, showed a significant inhibition of TPA-induced morphological changes in COLO205 cells (Fig. 4A). Additionally, PD, GF, H7, but not SB or SP, inhibits TPA-induced MMP-2 protein expression with a decrease in activity of MMP-2 enzyme released from COLO205 cells (Fig. 4B).

Moreover, PD, GF, and H7 significantly blocked TPA-induced ERK1/2 protein phosphorylation and c-Jun protein expression (Fig. 4C). These data suggested that activation of ERK1/2 located at downstream of PKC activation was followed by inducing c-Jun protein expression, and these events occurred at the upstream of MMP-2 activation induced by TPA.

ME suppression of TPA-induced MMP-2 activation is accompanied by inhibition of PKC translocation, ERK1/2 protein phosphorylation, and c-Jun protein expression in COLO 205 cells

The possibility that ME inhibited TPA-induced MMP-2 activity was further examined. Results shown in Fig. 5A indicated that ME significantly inhibited TPA-induced activity of MMP-2 enzyme released from COLO205 cells. In addition, TPA-induced PKC translocation, ERK1/2 protein phosphorylation, and c-Jun and MMP-2 protein expression were significantly blocked by ME, but not by MI. (Fig.5B). Addition of low concentrations of PD (1, 2, 4 μ M) or GF (0.5, 1, 2 μ M) enhanced the MMP-2 inhibition of ME (5 μ M) under TPA treatment (Fig. 5C). These data provided additional evidence that ME inhibited TPA-induced MMP-2 activity via blocking intracellular signaling transduction.

Anti-invasive effect of ME in COLO205 cells

We further investigated if ME blocked TPA-induced invasion in COLO205 cells. In vitro invasion assay using a transwell chamber coated with a reconstituted basement membrane (EHS matrigel) was performed to examine the anti-invasive effect of ME. In this assay system, COLO 205 cells were cultured on the upper chamber in the presence or absence of ME or MI (100 and 200 μ M), and TPA (100 ng/ml) was added to the lower chamber. After 24 hr incubation, COLO205 cells in the lower chamber were observed microscopically with (lower panel) or without (upper panel) Giemsa staining (Fig. 6A), and the number of viable cells in the lower chamber was quantified by MTT assay (Fig. 6B). Result shown in Fig. 6A indicated that COLO205 cells did not penetrate the EHS-coated filter in the absence of TPA in the lower chamber (control group). Addition of TPA induced invasion of COLO205 cells from the top to the lower chambers in the absence of ME or MI. Addition of ME, but not MI, significantly reduced TPA-induced invasion in COLO205 cells. Results of quantification of cells in the lower chambers supported that ME but not MI inhibited

TPA-induced invasion in COLO205 cells (Fig. 6B).

Discussion

Results of the present study demonstrate that ME significantly inhibits MMP-2 activity and tumor invasion in colorectal carcinoma cells. ME inhibition of MMP-2 activity was via a decrease in MMP-2 protein expression and a direct inhibition of MMP-2 enzyme activity. In the presence of TPA, ME suppressed PKC activation, ERK1/2 proteins phosphorylation, and c-Jun protein expression together with a reduction in MMP-2 protein expression and enzyme activity. Inhibition of TPA-induced tumor invasion by ME was demonstrated by in vitro invasion assay in the present study. These results suggest that ME, a compound capable of blocking both MMP2 protein expression and enzyme activity, is a potentially effective antitumor agent.

Flavonoids are known to possess several biological functions including anti-tumor, anti-oxidant, and anti-inflammation activities. Structure-activity analysis has shown that the number and location of OH substitutions are essential for the biological effects of flavonoids (19,20). Results of the present study indicated that ME significantly inhibited MMP-2 activity. However, quercetin, which is a potent antioxidant and an apoptotic inducer, did not suppress MMP-2 activities. In contrast to quercetin, ME contains an additional OH substitution at C4' of quercetin structure. Additionally, an OH group at C4' is also found in kaempferol and luteolin, both showed about 50 and 13 % inhibition of MMP-2 enzyme activity, respectively. These results suggest that OH at C4' of flavone is important for MMP-2 inhibition.

In addition, glycoside has been shown to affect the biological functions of flavonoids via increasing their hydrophilic properties. Our previous studies demonstrated that rutinose and rhamnoglucoside attenuated apoptosis-inducing activity and NO synthesis inhibition of flavonoids (14,15,21). In the present study, all the tested glycosylated compounds including quercitrin, MI, baicalin, rutin, naringin, and hesperedin did not inhibit activity of MMP-2 enzyme released from COLO205 cells. A significant difference was found in ME and its glycoside MI. MI, which contains a rhamnoside at C3 of ME, did not inhibit endogenous and TPA-induced MMP-2 enzyme activity and invasion. These data support the notion that glycosides are negative moieties on MMP-2 inhibition and invasion in colorectal carcinoma cells.

It has been shown that flavonoids containing more hydroxyl substitutions exhibit stronger antioxidant activity (22, 23). Wang et al (24) indicated that OH groups at C3'

and C4' of the B ring and a 2,3-double bond in conjugation with a 4-oxo group in the C ring, along with the polyphenolic structures were crucial for the protection from H₂O₂-induced oxidative stress. Cos et al (25) indicated the OH groups at C5 and C7, and a double bond between C2 and C3 were important for the inhibitory activity of flavonoids on xanthine oxidase activity. In contrast to OH substitutions, the methoxyl (OCH₃) addition inactivated both antioxidant and the prooxidant activities of the flavonoids, and hydroxylation, but not methoxylation, at C2 of flavone showed high affinity on benzodiazepine receptors (26, 27). In order to elucidate the relationship between the anti-oxidant activity and MMP-2 inhibition of flavonoids, anti-DPPH radical activity of indicated flavonoids was examined. It indicated that flavonoids with more than 2 OH substitutions such as quercetin, kaempferol, baicalein, ME, MI, and toxifolin exhibited potent anti-DPPH radical activity (Data not shown). However, results of the present study showed that ME but not others exhibited significant inhibition on MMP-2 enzyme activity. It suggests that ME inhibition of invasion and MMP-2 enzyme activity is not only due to its anti-oxidant activity but also due to its suppression of MMP-2 protein expression and enzyme activity via blocking intracellular signaling transduction processes.

TPA, a tumor promoter, has been used extensively in induction of tumor progression in vitro and in vivo. Activation of PKCs via translocation of PKCs from cytosol to membrane has been identified under TPA treatment (28). In addition to PKCs, phosphorylation of MAPKs including ERK1/2, p38, and JNK, and induction of c-Jun gene expression were detected in TPA-induced proliferation (8, 29). Hah et al (30) reported that TPA induced the invasion of the hepatocellular carcinoma cells through MMP-9 but not MMP-2 secretion. However, effect of TPA on MMP-2 enzyme activity and invasion in colorectal carcinoma cells is still undefined. Results of the present study indicated that TPA induced invasion and MMP-2 protein expression in colorectal carcinoma cells COLO205. Sequential induction of PKC

translocations, ERK 1/2 proteins phosphorylation, and c-Jun protein expression was involved. ME inhibited TPA-induced MMP-2 enzyme activity and invasion via blocking activated signaling cascades. These data suggested that addition of TPA was able to induce invasion via activation of MMP-2 enzyme activity in colorectal carcinoma cells, which was blocked by ME (Fig. 7).

Several natural products such as curcumin, epigallocatechin, and resveratrol have

been found to inhibit MMPs activity and invasion in different assay systems (31-33). The mechanism of inhibition was attributed to blockade of MMPs' gene expression but not enzyme activity. In the present study, ME inhibited both endogenous and TPA-induced MMP-2 enzyme activity and invasion in colorectal carcinoma cells. The most important event was that ME inhibition of MMP2 released from cells was through blocking both MMP-2 protein expression and enzyme activity. The inhibition by ME of MMP-2 protein expression may be due to interrupt signaling transduction cascades such as PKC translocations and ERK1/2 proteins phosphorylation. However, the reason for ME-inhibited MMP2 enzyme activity is still unclear. Several previous studies demonstrated the metal-chelating activity of flavonoids (34, 35). Therefore, it is suggested that a direct inhibitory effect of ME on MMP-2 enzyme activity may be through chelating metals such as zinc and calcium, which are essential for the activity of MMP-2.

In conclusion, we have provided evidence demonstrating that ME inhibits invasion, and both MMP-2 protein expression and enzyme activity in colorectal carcinoma cells in both un-stimulated and TPA-stimulated conditions. Therefore, ME is potentially a useful anti-invasive agent in the treatment of colorectal carcinoma.

Acknowledgements

This study was supported by grants from National Science Council (NSC 93-2321-B-038-009, NSC92-2320-B-038-021, NSC92-2321-B-038-007, NSC-92-2320-B-320-025, and NCS93-2745-B-320-004-URD).

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Legends

Figure 1. Differential inhibitory effect of myricetin (ME) and its glycoside myricitrin (MI) on MMP2 enzyme activity in human colorectal carcinoma cells. Fig A shows structures of ME and MI. The latter contains a rhamnose at C3 of ME. Fig B shows that ME but not MI inhibits activity of MMP-2 enzyme released from indicated colorectal carcinoma cells. Cells were treated with different concentrations (6.25, 12.5, 25, 50, 100, 200 μ M) of ME or MI for 24 hr, and 20 μ l of medium were applied for gelatin zymography. In Fig C, cytotoxicity of ME and MI in COLO 205 cells was evaluated by MTT assay as described in the section of MATERIALS AND METHODS. Data was expressed as the mean $\pm$ SE from three-independent experiments, and * P <0.05 indicates significantly different from control group as analyzed by Student's t -test. The intensity of each band was quantitated by densitometry analysis, and expressed as folds of control. Gelatin zymography was performed at least three times, and the results shown are representative of all of the data.

Figure 2. ME but not MI inhibits both MMP-2 protein expression and enzyme activity. (A) ME inhibits MMP-2 protein expression in COLO 205 cells. Cells were treated with ME or MI (100 or 200 μ M) for 24 hr, and expression of MMP-1, MMP-2, MMP-3 and MMP-9 protein in cells was detected by Western blotting using specific antibodies. (B) ME inhibits MMP-2 enzyme activity in the medium. The condition medium after 24 hr incubation of COLO205 cells was incubated with different concentrations (6.25, 12.5, 25, 50, 100, 200 μ M) of ME or MI at 37°C for 30 min. Activity of MMP-2 enzyme in each sample was analyzed by gelatin zymography. (C, D) ME-inhibited MMP-2 enzyme activity using purified MMP-2 protein as a substrate. Purified MMP-2 protein (500 ng) from human fibroblast was incubated with different concentrations (25, 50, 100 and 200 μ M) of ME (C) or MI (D) at 37°C for 30 min. Activity of MMP-2 enzyme in each sample was analyzed by gelatin zymography (upper panel). The amount of MMP-2 protein in each sample was detected by Western blotting (WB) using a specific anti-MMP2 antibody (middle panel). Quantification of MMP2 enzyme activity under different treatments were performed by densitometry analysis, and expressed as a fold of control (lower panel). Data was expressed as the mean $\pm$ SE from three-independent experiments, and ** P <0.01 indicates significantly different from control group as analyzed by Student's t -test. Gelatin zymography and Western blotting were performed at least three times, and the results shown are representative of all of the data.

Figure 3. TPA time-dependently induced MMP-2 protein and enzyme activity, c-jun protein, ERK1/2 proteins phosphorylation, and PKC α translocation in COLO 205 cells. (A) COLO

COLO205 cells were treated with TPA (50 ng/ml) for indicated time points, and MMP-2 activity in the medium and expression of indicated proteins including MMP-1, MMP-2, MMP-3, and MMP-9 proteins were analyzed by gelatin zymography and Western blotting, respectively. (B) TPA induced PKC α translocation from cytosol to membrane. Cells were treated by TPA for 30 min, and the fractions of membrane and cytosol were collected as described in MATERIALS AND METHODS. The expression of PKC α protein was detected by Western blotting. (C) TPA induced ERK1/2 but not p38 and JNK protein phosphorylation in COLO205 cells. Cells were treated with TPA (50 ng/ml) for different times, and expression of indicated proteins was detected by Western blotting using specific antibodies. ERK, p38, and JNK indicate the total amount of indicated proteins in cells, and p-ERK, p-p38, and p-JNK represent the respective phosphorylated proteins. Western blotting was performed at least three times, and the results shown are representative of all of the data.

Figure 4. Induction of PKC α translocation, ERK1/2 protein phosphorylation, and c-Jun protein expression involves in TPA-induced transformation and MMP-2 enzyme activity. (A) Activation of PKC α and ERK1/2 protein participated in TPA-induced transformation in COLO205 cells. Cells were treated with PD98059 (PD, 10 μ M, MEK inhibitor), SB203580 (SB, 10 μ M, p38 kinase inhibitor), SP600125 (SP, 10 μ M, JNK kinase inhibitor), GF-109203X (GF, 10 μ M, PKC inhibitor), or H7 (10 μ M, PKA and PKC inhibitor) for 30 min followed by incubating with TPA (50 ng/ml) for additional 24 hrs. The morphology of cell was detected microscopically with (Lower panel) or without (upper panel) Giemsa staining. (B) PD, GF, and H7 inhibited TPA-induced MMP2 protein expression and activity of enzyme released from COLO205 cells. As described in (A), MMP-2 protein (lower panel) and enzyme activity (upper panel) in cells and medium were analyzed by Western blotting and gelatin zymography, respectively. (C) PD, GF, H7 inhibited TPA-induced ERK1/2 protein phosphorylation and c-Jun protein expression in COLO205 cells as described in Fig A. Cells were pre-treated with PD (PD98059; 5, 10 μ M), GF (GF-109203X; 2.5, 5 μ M), or H7 (H7; 5, 10 μ M) for 30 min followed by adding TPA and incubated for additional 30 min for p-ERK detection or 6 hr for c-jun protein detection by Western blotting using specific antibodies. Western blotting was performed at least three times, and the results shown are representative of all of the data.

Figure 5. ME inhibits TPA-induced MMP-2 protein expression and enzyme activity via blocking PKC α translocation, ERK1/2 proteins phosphorylation, and c-Jun protein expression in COLO205 cells. (A) ME inhibited TPA-induced MMP2 protein expression and

enzyme activity in COLO 205 cells. Cells were treated with TPA (50 or 100 ng/ml) for 24 hr in the presence or absence of ME (100 or 200 μ M) pretreatment for 1 hr. The MMP-2 enzyme activity in the medium was analyzed by gelatin zymography. (B) ME inhibited TPA-induced PKC α translocation, ERK1/2 proteins phosphorylation, and c-Jun protein expression in COLO205 cells. Cells were pre-treated with ME or MI (100, 200 μ M) for 1 hr, followed by adding TPA (50 ng/ml). The expression of indicated proteins was detected by Western blotting using specific antibodies as described in Fig 4. (C) Addition of PD or GF enhances the MMP-2 inhibition of ME. Cells were treated with or without PD (1, 2, 4 μ M) or GF (0.5, 1, 2 μ M) for 30 min followed by addition of ME (5 μ M) and TPA for a further 24 hr. MMP-2 enzyme activity in medium was examined by gelatin zymography. Gelatin zymography and Western blotting were performed at least three times, and the results shown are representative of all of the data.

Figure 7. Anti-invasion effect of ME in vitro. (A) ME but not MI inhibited invasion of COLO205 cells. In vitro invasion assay using Transwells coated with EHS matrigel was performed. Cells in upper chambers were treated with or without ME or MI (100 or 200 μ M), and TPA (100 ng/ml) was added into the lower chambers for 24 hr. In the control group, no TPA added in the lower well. In upper panel in Fig A, the cells in the lower chambers were observed microscopically. In lower panel in Fig A, cells in the lower chambers were detected by Giemsa staining and observed microscopically. Fig B shows quantification of cells in the lower chambers, which was performed by MTT assay. Data was expressed as the mean $\pm$ SE from three-independent experiments, and ** P <0.01 indicates significantly different from TPA-treated group as analyzed by Student's t -test.

Figure 7. A proposed mechanism for ME in suppressing MMP-2 enzyme activity and that induced by TPA in colorectal carcinoma cells.