Celastrol Inhibits Breast Cancer Cell Invasion via Suppression of NF- κ B-mediated Matrix Metalloproteinase-9 Expression

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Key Words

Celastrol • MMP-9 • Cell migration • Cell invasion • Breast cancer

Abstract

Background/Aims: Metastasis is one of the main causes of death for patients with malignant tumors. Induction of matrix metalloproteinase (MMP)-9 is particularly important for the invasiveness of various cancer cells. Celastrol, a triterpenoid isolated from the traditional Chinese medicine, is known to inhibit the proliferation of a variety of tumor cells, including leukemia, glioma, prostate and breast cancer cells. In this study, we investigated the effect of celastrol on the migration and invasion of human breast carcinoma cells. **Results:** We observed that celastrol suppressed phorbol 12-myristate 13-acetate (PMA)-induced invasion and migration of MCF-7 cells. We also found that celastrol inhibited PMA-induced MMP-9 expression at both the mRNA and the protein levels, and the proteolytic activity of MMP-9 in MCF-7 cells. Our results revealed that celastrol inhibited the transcriptional activity of MMP-9 by suppression of the DNA binding activity of NF- κ B in the MMP-9 promoter, and inhibited degradation of I κ B α and

nuclear translocation of NF- κ B. **Conclusion:** These results indicate that celastrol inhibits NF- κ B-mediated MMP-9 expression, resulting in suppression of breast cancer cell invasion and migration that is induced by PMA. Celastrol is a potential agent for clinical use in preventing the invasion and metastasis of human malignant breast tumors.

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Introduction

Metastasis and invasion are fundamental properties of malignant cancer cells [1]. Therefore, the control of metastasis and invasion represents an important therapeutic target. There is evidence that increased expression of matrix metalloproteinases (MMPs) is involved in tumor invasion and metastasis in many cancer types [2]. Among the members of the human MMP family, MMP-2 and -9 are the key enzymes that are involved in degrading type-I and -IV collagens and extracellular matrix (ECM) [3, 4]. Generally, MMP-2 is constitutively overexpressed in highly metastatic tumors [5], whereas MMP-9 can be stimulated by cytokine and phorbol 12-myristate 13-acetate (PMA), through activation of

transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) [6-9]. It has been reported that the human MMP-9 promoter contains *cis*-acting regulatory elements and the transcription factor binding sites including NF- κ B (-600 bp) and AP-1 (-533 bp, -79 bp) binding sites, which participate in regulation of the MMP-9 gene transcription [10-12]. NF- κ B, a heterodimer of p50 and p65, is sequestered in the cytoplasm due to its association with the inhibitory protein I κ B α under normal conditions. Stimulation by PMA leads to the dissociation of I κ B α from NF- κ B and degradation through the ubiquitin/proteasome-dependent pathway [13, 14]. The released NF- κ B translocates into the nucleus and binds to the promoter region of MMP-9, leading to gene expression [13]. Therefore, upstream molecules that regulate MMP-9 expression and enzymatic activity can be used as a target for treating breast cancer metastasis.

Recent studies to detect new anti-metastatic agents have demonstrated that plant-derived compounds with chemopreventive potential inhibit the growth and metastasis of several types of cancer. Celastrol is a triterpenoid isolated from the traditional Chinese medicine, "Thunder of God Vine" (*Tripterygium wilfordii* Hook F.) that has been used in the treatment of autoimmune disease, asthma, chronic inflammation [15, 16]. Celastrol is also known to inhibit the proliferation of a variety of tumor cells, including leukemia [17, 18], glioma [19], prostate [20], and breast cancer cells [21]. Studies to define its therapeutic mechanism showed that it suppresses the tumor necrosis factor (TNF)-induced tumor cell invasion [17], inhibiting NF- κ B signaling [22], and suppresses heat shock protein 90 [23], and proteasome activity [20]. However, the molecular mechanism underlying the invasion and migration effects of celastrol is not yet fully understood in breast cancer. In this study, we investigated the effects of celastrol on PMA-induced MMP-9 expression in breast cancer cells and explored the underlying upstream signaling mechanism. We found that celastrol significantly inhibited the MMP-9 gene expression by suppressing the NF- κ B activation and consequently reduced invasion and migration of MCF-7 human breast cancer cells.

Materials and Methods

Cells and reagents

The human breast cancer cell line MCF-7 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and penicillin-streptomycin (100 μ g/ml) at 37°C with 5% CO₂ atmosphere in a

humidified incubator. Gelatin was obtained from DIFCO (Lexington, KY). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA). PMA was purchased from Calbiochem (La Jolla, CA). Anti-p65, anti-tubulin, and anti-I κ B α antibodies were from Santa Cruz (Santa Cruz, CA). Anti-p-I κ B α antibody was from Cell signaling (Beverly, MA). All the chemicals not included above were from Sigma.

Flow cytometry

Cells were plated at a density of 1×10^6 cells per 100-mm culture dish and incubated for 48 h. Cells were trypsinized and then centrifuged at $250 \times g$ for 5 min, fixed in 70% ethanol, and kept at -20°C. Fixed cells were incubated with RNase A (50 μ g/ml) for 25 min prior to staining nucleic acid with propidium iodide (50 μ g/ml) for 5 min. The sub G₁ value in each group was analyzed by flow cytometer (BD Bioscience).

Cell proliferation and viability assay

All proliferation assays were based on the MTT method. Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. After 24 and 48 h, cells were treated with various concentration of celastrol. At the end of the experiment, the media was removed and DMSO was added with MTT solubilization solution. Absorbance was measured at 550 nm.

Colony forming assay

MCF-7 cells were seeded into 6-well plate and allowed to attach for 24 h at 37°C in culture medium. Cells were then treated with various concentration of celastrol. After 4 days, colonies were fixed with fixing solution (methanol:acetic acid = 3:1) for 10 min at room temperature and stained with 0.01% crystal violet solution. Plates were washed with PBS and were photographed.

In vitro invasion assay

Matrigel invasion assays were used to assess the effect of celastrol. The 8- μ m pore size polycarbonate nucleopore filter inserts in a 24-well transwell chamber (BD Biosciences) were coated with 30 μ g/well matrigel (Sigma). Celastrol treated-MCF-7 cells were seeded into the upper part of the matrigel-coated filter, and serum-free RPMI with or without 100 nM PMA was added to the lower part. After 36 h, the cells that had migrated through the matrigel and the 8- μ m pore-size membrane were fixed, stained, and counted under a light microscope.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from cells with Trizol (Invitrogen) according to the manufacturer's protocol. Approximately 2 μ g of total RNA was used to prepare cDNA using the Superscript First Strand cDNA Synthesis Kit (Bioneer, Daejeon, South Korea). The experimental conditions were as follows: 96°C for 40 sec, 60°C for 40 sec, and 72°C for 40 sec for 22 cycles. The PCR products were electrophoresed on a 2% (w/v) agarose gel in 1 $\times$ Tris-acetate-EDTA (TAE) buffer, and stained with ethidium bromide solution. All the PCR reactions were repeated at least three times. The following primers were used in this study: 5'-TCC CTG GAG ACC TGA GAA CC-3' and 5'-CGG CAA GTC TTC CGA GTA GTT-3' for MMP-9; 5'-TGA GCT CCC GGA

Fig. 1. Celastrol suppresses PMA-induced proliferation and invasion of MCF-7 cells. (A) Chemical structure of celastrol. (B) MCF-7 cells were incubated with the indicated concentration of celastrol in serum-containing medium for 24 and 48 h, and the cell viability was measured using a MTT assay. * $p < 0.05$, ** $p < 0.01$ versus vehicle alone-treated cells. (C) MCF-7 cells were incubated with the indicated concentration of celastrol for 24 h. Cell were harvested and the cell cycle distribution in the sub G_1 phase was determined by FACS analysis. * $p < 0.05$, ** $p < 0.001$ versus vehicle alone-treated cells. (D) MCF-7 cells were pretreated with the indicated concentration of celastrol for 30 min and then stimulated with 100 nM PMA for 24 h. After 24 h, colonies were fixed with fixing solution for 10 min at room temperature and stained with 0.01% crystal violet solution. Representative photographs demonstrating colony formation are shown. Original magnification was $\times 100$. Scale bars, 100 μm . The number of colonies from triplicate plates was quantified using Image J. # $p < 0.01$ versus vehicle alone-treated cells, * $p < 0.01$, ** $p < 0.001$ versus PMA alone-treated cells. (E) MCF-7 cells were treated with the indicated concentration of celastrol for 36 h in the absence or presence of 100 nM PMA. The invasion ability of MCF-7 cells was determined by a Matrigel invasion assay. Original magnification was $\times 200$. Scale bars, 100 μm . The cell invasion ability was quantified. # $p < 0.001$ versus vehicle alone-treated cells, * $p < 0.01$, ** $p < 0.001$ versus PMA alone-treated cells. Data represent the mean $\pm$ SE of three independent experiments.

AAA GAT TG-3' and 5'- TCA GCA GCC TAG CCA GTC G-3' for MMP-2; 5'- GGG GCT TCA CCA AGA CCT ACA C-3' and 5'-AAG AAA GAT GGG AGT GGG AAC A-3' for TIMP-1; 5'- CCA TCA CCA TCT TCC AGG AG-3' and 5'- CCT GCT TCA CCA CGT TCT TG-3' for GAPDH. GAPDH was amplified as an internal control. The intensity of each band amplified by RT-PCR was analyzed using MultiImageTM Light Cabinet (version 5.5, Alpha Innotech Corp., San Leandro, CA), and normalized to that of GAPDH mRNA in corresponding samples.

Gelatin zymography

The presence of MMP-9 in the supernatants of DMSO or celastrol treated MCF-7 cells was analyzed with gelatin zymograms. Briefly, cells were incubated in serum-free RPMI and the supernatants were collected after incubation for 24 h, clarified by centrifugation, normalized to cell number, mixed with non-reducing Laemmli sample buffer, and separated by

electrophoresis in 10% SDS-PAGE containing 1 mg/ml gelatin (DIFCO). After electrophoresis, gels were re-natured by washing in 2.5% Triton X-100 solution twice for 30 min to remove all SDS. The gels were then incubated in 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L CaCl_2 , and 1 μM ZnCl_2 at 37°C overnight. After incubation, the gels were stained with 0.05% Coomassie brilliant blue R-250 for 30 min at room temperature and then destained in distilled water. MMP-9 activities were visible as clear bands on a blue background where the gelatin substrate had been hydrolyzed by enzyme activity.

ELISA

The supernatants were collected for measuring secreted-MMP-9 protein. The total and active MMP-9 protein was assayed according to SensoLyte PlusTM 520 MMP-9 assay system (AnaSpec, San Jose, CA). MMP-9 activity unit was expressed as a change in fluorescence intensity at excitation of 490 nm/emission of 520 nm.

Fig. 2. Celastrol reduces PMA-induced expression and proteolytic activity of MMP-9. (A) MCF-7 cells were incubated with the indicated concentration of celastrol for 30 min followed by 100 nM PMA stimulation for 24 h. After 24 h, the mRNA level of endogenous MMP-9, TIMP-1, and MMP-2 were measured by RT-PCR and GAPDH was used as an internal control. (B) MCF-7 cells were incubated with celastrol and/or PMA (100 nM) for 24 h. MMP-9 expression was analyzed by real-time PCR. GAPDH was used as internal control. $^{\#}p < 0.001$ versus vehicle alone-treated cells, $^{*}p < 0.05$ versus PMA alone-treated cells. (C and D) MCF-7 cells were pretreated with celastrol for 30 min and stimulated with 100 nM PMA for 24 h. After 24 h, the conditioned medium was collected and assayed for the amount and the activity of secreted MMP-9 using ELISA and gelatin zymography. $^{\#}p < 0.001$ versus vehicle alone-treated cells, $^{**}p < 0.001$ versus PMA alone-treated cells. Data represent the mean $\pm$ SE of three independent experiments.

Wound healing assay

The transfected MCF-7 cells were incubated until 90% to 100% confluent. After cells were scratched by P-10 pipette tip, cells were incubated for various time periods. The pictures were taken at 0, 6, 12, and 24 h. Phase contrast images were taken by Nikon microscopy system (Nikon Instrument). Measurement of the wound-healing gap distance was performed using the computer program Image J. Results were expressed as the mean $\pm$ SE. $P = 0.05$ was considered significant (Student's *t* test).

Transient transfection and luciferase reporter assay

The transcriptional activities of MMP-9, NF- κ B, and AP-1 were measured by luciferase reporter assay using the pMMP-9-Luc, and pNF- κ B-Luc reporter plasmids. MCF-7 cells were seeded into 6-well plates. Cells at 70-80% confluence were co-transfected with 0.2 μ g of MMP-9, or NF- κ B reporter constructs and 0.2 μ g pSV- β -galactosidase for 24 h. The transfected cells were incubated with celastrol and then stimulated with 100 nM PMA for 9 h. Luciferase and β -galactosidase activities were assayed according to the manufacture's protocol (Promega), using a Luminometer 20/20n (Turner BioSystems, Sunnyvale, CA). Luciferase activity was normalized by β -galactosidase activity in cell lysate and expressed as an average of three independent experiments.

Cell fractionation and Immunoblotting

Nuclear and cytosolic fractions were prepared using Nuclear and Cytosolic Extraction Reagents kit (Fermentas). PCNA and α -tubulin were used as markers for nuclear and

cytosol proteins, respectively. Lysate proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with TBS containing 0.1% Tween 20 and 5% skim milk, and then exposed to the desired primary antibodies. After treatment with a proper secondary antibody, the immunoreactive bands were visualized by standard ECL method.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing the NF- κ B (5'-AGT GAG GGG ACT TTC CCA GGC-3') or AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') consensus sequences were 5'-end-labeled with γ - 32 P ATP using T4 polynucleotide kinase. Unincorporated nucleotide was removed by passage over a Bio-Gel P-6 spin column (Bio-Rad, Inc., Hercules, CA). Nuclear extract was incubated with radiolabeled probe for 20 min, and protein-DNA complexes were separated from free probes by electrophoresis on a 4% native polyacrylamide gel in 0.5 $\times$ Tris-HCl (pH 7.5), 1 mM MgCl₂, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 50 mg/ml of poly (di-dC). Dried gels were visualized by autoradiography.

Statistics analysis

Statistical analysis was performed by computer program Prism (GraphPad Software, La Jolla, CA). The results are presented as the mean $\pm$ S.E. The statistical significance of differences between groups was analyzed via repeated measures of one-way analysis of variance followed by Student's *t*-test. Data were considered statistically significant when $p < 0.05$.

Fig. 3. Celastrol inhibits PMA-induced migration of MCF-7 cells. (A and B) MCF-7 cells were scratched with a pipette tip and were incubated with various concentrations of celastrol and 5 nM MMP-9 inhibitor (MMP-9 I). After 30 min, cells were treated with 100 nM PMA for 24 h. Representative images of wound healing were taken on the time of the scratch and 24 h after the wound scratch. Original magnification was $\times 100$. Scale bars, 100 μm . The level of cell migration into the wound scratch was quantified as the percentage of wound healing. $**p < 0.001$ versus vehicle alone-treated cells, $*p < 0.05$ versus PMA alone-treated cells. Data represent the mean $\pm$ SE of three independent experiments.

Results

Celastrol suppresses PMA-induced invasion of MCF-7 cells

It has been reported that celastrol is involved in breast cancer cell proliferation (Fig. 1A) [21]. To investigate the pharmacological potential of celastrol in breast cancer, we first examined the effect of celastrol on cell proliferation in breast cancer cell line MCF-7. MCF-7 cells were treated with various concentrations of celastrol in serum-containing medium for 24 and 48 h, and determined cell viability using MTT assay. Celastrol slightly decreased cell viability at a concentration of 1 μM , however, 2.5 μM celastrol decreased cell viability by approximately 2-3 fold (Fig. 1B). To further explore the effect of celastrol on cell cycle, we monitored a cell cycle profile using fluorescence-activated cell sorting (FACS) analysis. Celastrol did not influence the cell cycle transition at low dosage. However, a high dosage of celastrol triggered the sub G1 accumulation (Fig. 1C). To investigate the inhibitory effect of celastrol on cell proliferation, we performed a colony formation assay. PMA used as a positive stimulator markedly enhanced colony forming ability of MCF-7 cells, compared with control cells (Fig. 1D). Cells treated with celastrol at low dosage slightly suppressed PMA-induced colony formation, whereas 2.5 μM celastrol completely inhibited colony forming ability of MCF-7 cells (Fig. 1D). We next examined whether celastrol inhibits PMA-induced

invasion in MCF-7 cells. Cells treated with PMA caused a 12-fold increase in invasion of MCF-7 cells, however, PMA-induced cell invasion was inhibited by celastrol in a dose-dependent manner (Fig. 1E). Interestingly, celastrol significantly inhibited PMA-induced cell invasion at non-cytotoxic dosage (0.5 μM or less), indicating that celastrol is an effective inhibitor of cell migration, invasion, and metastasis in MCF-7 breast cancer cells.

Celastrol reduces PMA-induced expression and proteolytic activity of MMP-9

MMP-9 and MMP-2 are important ECM-degrading enzymes [1]. It has been reported that both enzymes were involved in cancer cell invasion and metastasis [10]. The fact that celastrol inhibited cancer cell invasion prompted us to examine the effect of celastrol on MMPs gene expression. As shown in Fig. 2A, PMA induced MMP-9 mRNA expression in MCF-7 cells, whereas treatment with celastrol suppressed PMA-induced MMP-9 gene expression in a dose-dependent manner. However, the mRNA expressions of MMP-2 and TIMP-1 were not affected by PMA and celastrol treatment (Fig. 2A and B). We next examined the effects of celastrol on the expression and secretion of MMP-9 protein, and the proteolytic activity of MMP-9 in the conditioned media. Results from ELISA and gelatin zymography revealed that celastrol inhibited the secretion and proteolytic activity of MMP-9 that was induced by PMA (Fig. 2C and D). These results indicate that celastrol selectively suppresses

Fig. 4. Celestrol inhibits the transcriptional activity of MMP-9 via suppression of PMA-stimulated NF- κ B activity. (A) MCF-7 cells were transfected with the pMMP-9-luciferase and the pSV40- β -galactosidase vectors. The transfected cells were treated with the indicated concentrations of celestrol for 30 min and stimulated with 100 nM PMA for 9 h. The luciferase activity was normalized by β -galactosidase activity. Each value represents the mean $\pm$ SD of three-independent experiments and is expressed relative to a control. (B and C) MCF-7 cells were transfected with the deletion mutants MMP-9-Luc and NF- κ B binding site mutant (mNF- κ B) MMP-9-Luc. The transfected cells were treated with celestrol for 30 min and stimulated with 100 nM PMA for 9 h. The luciferase activity was normalized by β -galactosidase activity. Each value represents the mean $\pm$ SD of three-independent experiments and is expressed relative to a control. One-way ANOVA was performed to determine statistical significance (* p < 0.05). (D and E) MCF-7 cells were transfected with the reporter plasmids containing tandem NF- κ B or AP-1 binding sites. After 24 h, cells were treated with or without 100 nM PMA for 9 h and the luciferase activities were determined. Each value represents the mean $\pm$ SD of three-independent experiments and is expressed relative to a control. One-way ANOVA was performed to determine statistical significance (* p < 0.05).

PMA-induced MMP-9 expression at both the protein and mRNA levels, and subsequently inhibited the enzymatic activity of MMP-9.

Celestrol inhibits PMA-induced migration of MCF-7 cells

Since celestrol inhibited PMA-induced invasion of MCF-7 cells, we performed a wound healing assay using MMP-9 inhibitor in order to examine the involvement of MMP-9 in breast cancer cell migration. Results showed that MMP-9 inhibitor blocked PMA-induced wound healing ability of MCF-7 cells, indicating that MMP-9 is largely responsible for PMA-induced migration of MCF-7 cells (Fig 3A and B). Celestrol also inhibited PMA-induced migration of MCF-7 cells in a dose-dependent manner (Fig. 3A and B). These results indicate that celestrol reduces cell migration by suppressing MMP-9 expression and secretion in PMA-treated MCF-7 cells.

Celestrol inhibits the transcriptional activity of MMP-9 via suppression of PMA-stimulated NF- κ B activity

NF- κ B and AP-1 play important roles in controlling MMP-9 expression in various cancer cell lines [8]. Since celestrol induces the mRNA expression of MMP-9, we examined whether celestrol affects the promoter activity of MMP-9. Luciferase reporter gene containing the MMP-9 promoter region (-925/+13) was transiently transfected into MCF-7 cells and the luciferase activity was determined. As shown in Fig. 5A, the promoter activity of MMP-9 in cells treated with PMA was up-regulated by 3-fold, compared with control cells. Celestrol inhibited PMA-induced promoter activity of MMP-9 in a dose-dependent manner (Fig. 4A), indicating that celestrol inhibits MMP-9 expression at the transcriptional level. The transcription factor binding sites in the MMP-9 promoter include NF- κ B (-600 bp), SP-1 (-569 bp) and

Fig. 5. Celastrol inhibits the DNA binding activity of NF- κ B in the MMP-9 promoter. (A and B) MCF-7 cells were pretreated with the indicated amounts of celastrol for 30 min and stimulated with 100 nM PMA for 30 min. Nuclear extracts were prepared and incubated with radiolabeled oligonucleotides containing the NF- κ B and AP-1 motif in the MMP-9 promoter. (C) MCF-7 cells were pretreated with the indicated amounts of celastrol for 30 min and stimulated with 100 nM PMA for 30 min. Cells were harvested and fractionated into the cytoplasm and the nucleus. Lysates were then separated on a 10% SDS-polyacrylamide gel and subjected to Western blotting with anti-p65, anti-p-I κ B α , and anti-I κ B α antibodies. The analysis was repeated in three times, and β -actin and PCNA were used as markers for the cytoplasmic and nuclear fractions. The NF- κ B protein level that was translocated to the nucleus was quantified by densitometric analyses. The bars represent the mean $\pm$ SD. One-way ANOVA was performed to determine statistical significance (* $p < 0.05$).

AP-1 (-533 bp and -79 bp) binding sites. To determine the transcription factors that participate in regulation of MMP-9 transcription, we generated the deletion mutants containing different transcript factor binding sites in the MMP-9 promoter. Although celastrol reduced PMA-induced promoter activity of MMP-9 by 3-fold, the promoter activities of NF- κ B deletion mutants (-580/+13 and -450/+13) were slightly affected by celastrol (Fig. 4B). These results indicate that the NF- κ B (-600 bp) binding site in the MMP-9 promoter is involved in the inhibitory effect of celastrol on PMA-induced transcriptional activation of MMP-9. To confirm these findings, we generated the promoter with mutation in the NF- κ B (-600 bp) binding site. Results showed that PMA-induced promoter activity of mutated promoter in the NF- κ B binding site (-925/+13) was not affected by celastrol (Fig 4C). To further investigate this observation, the luciferase reporter vectors containing tandem repeats of

the NF- κ B or AP-1 binding sites were used. As shown in Fig. 4D and E, the luciferase activity in cells transfected with the NF- κ B reporter was reduced in a dose-dependent manner due to celastrol treatment, whereas no significant changes were observed in cells transfected with the AP-1 reporter. These results indicate that NF- κ B transcription factor and NF- κ B binding sites in the MMP-9 promoter region contribute to the inhibitory effect of celastrol on PMA-induced MMP-9 transcription.

Celastrol inhibits the DNA binding activity of NF- κ B in the MMP-9 promoter

To determine the effect of celastrol on the DNA binding activities of NF- κ B and AP-1, we performed electrophoretic mobility shift assay. As shown in Fig. 5A and B, PMA induced the DNA binding activities of both NF- κ B and AP-1. However, celastrol significantly decreased PMA-induced DNA binding ability of NF- κ B,

whereas the DNA binding ability of AP-1 was not affected by celastrol. NF- κ B is sequestered in the cytoplasm by binding to the I κ B family and is activated by I κ B α phosphorylation and subsequent degradation in the proteasome, thus allowing NF- κ B subunits including p65 and p50 to enter the nucleus and activate the target genes [24]. We examined whether celastrol affects the phosphorylation of I κ B α and the nuclear translocation of NF- κ B p65 subunit. Results showed that PMA increased the phosphorylation of I κ B α in cytoplasm and the nuclear translocation of NF- κ B p65 subunit (Fig. 5C). However, celastrol inhibited PMA-induced phosphorylation of I κ B α in the cytoplasm in a dose-dependent manner (Fig. 5C). The enhanced level of NF- κ B p65 subunit in the nucleus due to PMA was also decreased by celastrol (Fig 5C). These results indicate that celastrol inhibits NF- κ B activation by suppressing I κ B α phosphorylation and the nuclear translocation of NF- κ B in PMA-treated MCF-7 cells.

Discussion

Celastrol, extracted from the celastraceae family of plants, is of particular interest because of its superior anti-tumor capabilities against a variety of tumors, including leukemia, prostate, breast, and pancreatic cancers, through modulation of proteasome activity [20], heat shock response [23, 25], estrogen receptor α [21], and NF- κ B signal pathway [17]. Although several reports mentioned about the possible involvement of celastrol in tumor metastasis, no study has been reported the anti-metastatic function of celastrol. Therefore, the molecular mechanism of anti-metastatic function of celastrol remains to be established. In the present study, we investigated the inhibitory effect of celastrol on PMA-induced invasion in MCF-7 cells. We demonstrated that: (a) celastrol inhibits PMA-induced invasion and migration, (b) celastrol regulates MMP-9 expression and activity, (c) celastrol specifically inhibits the DNA binding activity of NF- κ B, and (d) consequently, celastrol inhibits PMA-induced invasion and migration via suppression of NF- κ B-mediated matrix metalloproteinase-9 expression in breast cancer cells.

Tumor metastasis is a multi-step and complex process that includes cell proliferation [3], proteolytic digestion of ECM [1], cell migration to circulation system [5], and tumor growth at metastatic sites [26]. In particular, the overexpression of MMP-9 has been associated with the progression and invasion of different types of tumors

Fig. 6. Diagram of the signal pathway that celastrol inhibits PMA-induced breast cancer cell invasion and migration.

including mammary tumor [27]. PMA has been used as a tumor promoter in chemical-induced carcinogenesis *in vitro* and *in vivo*. Previous studies showed that PMA is able to promote tumor migration and invasion by stimulating MMP-2 or MMP-9 expression in glioma, colon, hepatoma, and breast cancer cells [28, 29]. However, the mechanism by which PMA induces breast cancer cell invasion has not been known. We found that PMA induced the invasion of MCF-7 breast cancer cells via induction of MMP-9 gene expression at both the mRNA and protein levels. We also found that invasion of MCF-7 cells due to PMA was blocked by celastrol. The inhibitory effect of celastrol on the MMP-9 enzyme activity and the secreted MMP-9 protein level in conditioned medium indicates that celastrol is involved in the transcriptional regulatory pathways responsible for MMP-9 gene expression.

The MMP-9 promoter region contains the cis-regulatory element, including one NF- κ B and two AP-1 binding sites, which are absent in the promoter region of MMP-2 [10, 11]. Due to the different structures of 5'-flanking regulatory sequences of MMP-2 and MMP-9 genes, the MMP-9 protein is highly inducible. Our results showed that MMP-9 mRNA transcription was markedly induced by PMA stimulation, leading to increased protein secretion and enzyme activity. However, PMA did not stimulate MMP-2 transcription. Since we were interested in the inhibitory mechanism of celastrol in MMP-9 expression, we examined the MMP-9 promoter activity using the deleted and mutated reporter plasmids. We found that the NF- κ B binding site is necessary for inhibition of

MMP-9 expression by celastrol in PMA treated MCF-7 cells. Furthermore, results from the luciferase assay indicate that celastrol suppresses tandem-NF- κ B promoter activity, whereas AP-1 had little effect on the MMP-9 transcriptional regulation by celastrol. Results from EMSA showed that AP-1 and NF- κ B are involved in PMA-stimulated MMP-9 gene expression. However, celastrol was only able to inhibit the DNA binding ability of NF- κ B but not AP-1. It has been reported that NF- κ B plays a regulatory role in PMA-induced MMP-9 expression in breast cancer cells [14]. Our results show that the NF- κ B pathway is the main regulatory pathway in suppression of PMA-induced MMP-9 gene expression by celastrol.

In conclusion, we found that celastrol inhibits breast cancer cell migration and invasion by suppressing MMP-9 expression and proteolytic activity via blocking the I κ B α degradation and NF- κ B activation (Fig. 6). Therefore,

celastrol is a potential agent for clinical use in preventing the invasion and metastasis of human malignant tumors.

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