RESEARCH ARTICLE

Curcumin Inhibits TGF-β1-Induced MMP-9 and Invasion through ERK and Smad Signaling in Breast Cancer MDA-MB-231 Cells

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Abstract

**Objective:** To evaluate the effects of curcumin on matrix metalloproteinase-9 (MMP-9) and invasion ability induced by transforming growth factor-β1 (TGF-β1) in MDA-MB-231 cells and potential mechanisms. **Methods:** Human breast cancer MDA-MB-231 cells were used with the CCK-8 assay to measure the cytotoxicity of curcumin. After treatment with 10 ng/ml TGF-β1, with or without curcumin (≤10 μM), cell invasion was checked by transwell chamber. The effects of curcumin on TGF-β1-stimulated MMP-9 and phosphorylation of Smad2, extracellular-regulated kinase (ERK), and p38 mitogen activated protein kinases (p38MAPK) were examined by Western blotting. Supernatant liquid were collected to analyze the activity of MMP-9 via zymography. Following treatment with PD98059, a specific inhibitor of ERK, and SB203580, a specific inhibitor of p38MAPK, Western blotting and zymography were employed to examine MMP-9 expression and activity, respectively. **Results:** Low dose curcumin (≤10 μM) did not show any obvious toxicity to the cells, while 0-10 μmol/L caused a concentration-dependent reduction in cell invasion provoked by TGF-β1. Curcumin also markedly inhibited TGF-β1-regulated MMP-9 and activation of Smad2, ERK1/2 and p38 in a dose- and time-dependent manner. Additionally, PD98059, but not SB203580, showed a similar pattern of inhibition of MMP-9 expression. **Conclusion:** Curcumin inhibited TGF-β1-stimulated MMP-9 and the invasive phenotype in MDA-MB-231 cells, possibly associated with TGF-β1/Smad and TGF-β1/ERK signaling.

**Keywords:** Curcumin - breast cancer - TGF-β1 - MMP-9 - MAPKs - invasion

Introduction

Breast cancer is one of most common malignant tumor in global female. And the leading cause of morbidity and mortality in breast cancer patients is cancer metastases, but not the primary tumor (Porter, 2009; Sanchez-Zamorano et al., 2011). It has been demonstrated that tumor cell invasion is the key event in the metastatic steps (Geiger et al., 2009). However, recent studies show that cancer progression toward metastasis is not entirely dependent on tumor cells themselves, but the result of the interactions of tumor cells and the primary tumor microenvironment (Allen et al., 2011). Cancer cells, specially those at the edges of the tumor, can attract and stimulate host cells to secret many kinds of factors (TGF-β1, IL-1, TNF-α, etc.) in the tumor microenvironment. Those factors can generate a series of invasion-related biological effects through the tumor cell signal transduction pathways, and then cause the cells to separate from the primary lesion focus and result in local invasion and distant metastasis finally (Allen et al., 2011). So far, novel findings indicates that TGF-β1 is one of the factors which could be highly relevant to breast cancer invasion and metastasis (Perera et al., 2010).

TGF-β1 is known to be one of the TGF-β superfamily members that plays a dual role in breast cancer generation and progression. In the early stages, TGF-β1 acts as a tumor suppressor, nevertheless, it promotes cancer invasion and metastasis in later stages (Biswas et al., 2007). After binding to TGF-β1, the type II TGF-β1 receptor recruits and phosphorylates the type I receptor. The activated type I receptor subsequently initiates the activation of Receptor-associated Smads (R-Smads), then the latter forms heteromeric complexes with common mediator Smad4 (co-Smad4) and translocate into the nucleus. The Smad complexes regulate specific target genes by directly or indirectly combining and interacting with transcription factors, co-repressors, and co-activators (Smith et al., 2012). In addition to the classical TGF-β1/Smad signaling pathway, TGF-β1 can also directly activate non-Smad signaling pathways (Zhang, 2009). Previous reports have shown the direct function of MAPK pathways, including c-Jun N-terminal kinase (JNK), extracellular- regulated kinase ERK, and p38 MAPK, in

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signal transduction of TGF-β1-regulated cell migration and invasion (Safina et al., 2007).

Researchers have demonstrated that the serum TGF-β1 level in early-stage breast cancer patients is uplifted and the high level of TGF-β1 has positive correlation with the effects of anti-tumor. However, it accelerates cancer invasion and formation of metastases in late-stage breast cancer (Cheung, 2007). Serra R et al. have found that radiation and chemotherapy can quicken tumor cells diffusion in mouse models of breast cancer. The experimental data indicate that the levels of TGF-β1 in those mouse models have been raised remarkably. In contrast, there is no far metastasis in the mouse models with low level of TGF-β1 (Serra et al., 2005). Therefore, antagonism of TGF-β1 signaling may provide a therapeutic target for late-stage breast cancer, blocking metastasis without detrimental side effects.

Curcumin is a natural phenolic pigment extracted from the roots of turmeric. Numerous studies have reported that it has positive pharmacological effects, such as anti-tumor, anti-oxidation, anti-inflammation, anti-rheumatism and so on (Yan et al., 2012). The U.S. National Cancer Institute has listed it as the third-generation anti-cancer drug to study, so that curcumin will likely to be a promising clinical anti-cancer drug (Park et al., 2008). Nevertheless, the precise molecular mechanisms underlying its anti-tumor invasion and metastasis are not entirely clear (Park et al., 2008). Most of the reports pay more attention to the anti-fibrosis activity of curcumin on TGF-β1-stimulated organs (Tubulointerstitial, corneal, liver, etc.) fibrosis via blocking TGF-β signaling pathway (Smith et al., 2010; Yao et al., 2012; Zhang et al., 2012). Kim et al. have confirmed that curcumin inhibits TGF-β1-induced MMPs in mouse keratinocytes (Santibáñez et al., 2000; Santibáñez et al., 2002), but the effects on breast cancer MDA-MB-231 cells have not been reported.

Therefore, the present study investigated the effect of curcumin on the exogenous TGF-β1–stimulated expression and activity of MMP-9 in human breast cancer MDA-MB-231 cells. Furthermore, the underlying mechanisms were also probed.

## Materials and Methods

### Cell culture

MDA-MB-231 cells were incubated with RPMI-1640 medium (Gibco, California, USA) containing 10% fetal calf serum at 37°C in 5% CO₂ incubator. Cells were cultured in serum-free media for 24h to synchronize cell growth before the experiments, then the media were exchanged for fresh serum-free medium, treated with various agents at the concentrations specified, and cells were harvested at different time points for various analyses.

### Cytotoxicity assay

MDA-MB-231 cells were trypsinized and seeded in 96-well plates at 5 × 10⁴ cells/well. After 24 h, escalating doses of curcumin (Sigma, USA) were added, and incubated for another 24 h, 48h, and 72h respectively. Cells without any treatment were used as control. Then, 10 μL CCK-8 (Dojindo, Tokyo, Japan) solution in culture medium was added to each well. Plates were incubated for an additional 2h. The optical density of each well was measured using microplate absorbance reader at a 450 nm wavelength. Cell viability was calculated as follows. The cell survival rate (%) = (A Treatment group - A blank wells) / (A negative control group - A blank wells) × 100%.

### Invasion assay

The MDA-MB-231 cells invasion behavior with or without indicated treatment was tested by Matrigel transwell system as described previously (Ye et al., 2012). After cultured in 6 well plates for 48h, the cells in different experimental groups were trypsinized, centrifuged, and resuspended at 1×10⁶ cells/mL in serum-free medium respectively. 100μl cell suspension per well were seeded onto the upper wells of transwells (8-μm-diameter pores; Millipore), which precoated with Matrigel (0.5 mg/MI, BD Biosciences Discovery Labware). Lower chamber of the transwells contained the medium containing 10% FBS as chemoattractant. After 8h of incubation, the cells on the upper chamber were carefully wiped with the cotton swab. The wells were washed 3 times with PBS, then fixed with 4% paraformaldehyde, and stained with crystal violet solution (Sigma Chemical, USA). The cells on the lower surface of the membrane were counted under a light microscope (magnification, ×100). The experiments were performed three times, each time in triplicate.

### Gelatin zymography

Cells in the logarithmic phase were seeded in 6-well plate at the density of 3 × 10⁵ cells per well. After incubated in serum-free medium with or without curcumin (2.5, 7.5 and 10 μM) and 10 μM TGF-β1 treatment for 48h. The supernatants were collected, and gelatin zymography assay was performed as described formerly (Zayani et al. 2012). After electrophoresis, the gels were washed three times with renaturing buffer containing 50 mM Tris–HCl, 5 mM CaCl₂ and 2.5% Triton X-100 (v/v), pH 7.5 for 30 min, followed by a brief rinsing with washing buffer (50 mM Tris–HCl , 5 mM CaCl₂), pH 7.5. Then the gels were incubated at 37°C for 42 h in developing buffer containing 50 mM Tris–HCl, 5 mM CaCl₂, 0.2 M NaCl, and 0.02% Brij 35, pH 7.5. The gels were subsequently stained with 0.25% Coomassie Brilliant Blue (G250) followed by destaining with a solution containing 10% acetic acid and 20% methanol. Enzyme-digested regions were visualized as light bands against a dark background. Zones of enzymatic activity were regarded as negatively stained bands.

### Western blotting

Cells in experimental groups were collected and lysed in RIPA buffer respectively. Supernatants of the cell lysates were used in the western blot analysis for MMP-9 (Bios, Beijing, China), β-actin (Beyotime, Beijing, China), and phosphorylation levels of ERK1/2, Smad2, p38MAPK (Cell signing technology, USA). After electrophoresis and transmembrane, the PVDF membranes (Millipore, USA) containing the proteins were blocked with 5% bovine serum albumin in TBST buffer (0.01%
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Figure 1. The Cell Toxicity of MDA-MB-231 Cells Treated by Curcumin. *P<0.05 vs. 0 μM Cur (24h), **P<0.05 vs. 0 μM Cur (48h), *P<0.05 vs. 0 μM Cur (72h)

Figure 2. Effect of Curcumin on TGF-β1-induced Invasion Ability of MDA-MB-231 Cells in vitro (crystal violet ×100) (a) MDA-MB-231 cells were incubated with different doses of Cur , with or without TGF-β1 (10 ng/ml) for 48h, then checked invasion ability by Transwell assay. (b) Quantitation of the cells which invasive matrix to lower surface of the membrane by cell counting under microscope (×100) (mean±SEM from 3 independent tests).*P<0.05 vs. control, **P<0.05 vs. TGF-β1 alone

Figure 3. Effect of Curcumin on MMP-9 Protein Expression Activated by TGF-β1 (a, b) Cell were incubated with different doses of curcumin and with or without TGF-β1 (10 ng/ml) for 48h, and the MMP-9 Protein expression was checked by Western blotting. (mean ± SEM from 3 separate tests) *P<0.05 vs. control, **P<0.05 vs. TGF-β1 alone. (c, d) MDA-MB-231 cells were treated with 10μM Cur, followed by TGF-β1 for different times, and the MMP-9 Protein expression was analyzed by Western blotting (mean ± SEM from 3 separate tests) *P<0.05 vs. control, **P<0.05 vs. TGF-β1 alone

CCK-8 assay. As shown in Figure 1, low-dose curcumin (≤10 μM) did not affect the viability of MDA-MB-231 cells, and survival rates of all the low-dose groups had exceeded 90% (except 10 μM curcumin for 72 h). But when the concentrations was above 10μM, curcumin time- and dose- dependently inhibited the growth of MDA-MB-231 cells. Therefore, the cells were treated with selected doses (≤ 10μM) for no more than 48 hours in subsequent experiments.

**Results**

The cytotoxicity effect of curcumin on the growth of MDA-MB-231 cells

To evaluate cytotoxicity of curcumin on the growth of breast cancer cells, MDA-MB-231 cells were treated with 5, 10, 15, 20, 30 and 50 μM curcumin for 24h, 48h, 72h respectively and then cell viability was detected by CCK-8 assay. The results showed that curcumin had a dose-dependent inhibitory effect on the cell growth of MDA-MB-231 cells. The cell viability was reduced in a concentration-dependent manner, with the strongest inhibitory effect at 50 μM curcumin.

Effects of curcumin on TGF-β1-induced invasiveness of MDA-MB-231 cells

We next examined the effect of curcumin on TGF-β1-induced cell invasion in MDA-MB-231 breast cancer cells using the transwell chamber assay. Our results showed that curcumin significantly reduced the invasiveness of MDA-MB-231 cells in a dose-dependent manner (Figure 1). This suggests that curcumin can prevent the TGF-β1-induced invasion in MDA-MB-231 cells.

Effects of curcumin on TGF-β1-Mediated MMP-9 protein expression and activity in MDA-MB-231 cells

We examined whether curcumin involved with the TGF-β1-induced MMP-9 protein expression. After pretreatment with different concentrations of curcumin for 30 min, the cells were cultured with TGF-β1 and various doses of curcumin for 48h. Western blot analyses revealed that the level of MMP-9 protein expression was significantly decreased by curcumin in a dose-dependent manner. The level of MMP-9 protein expressions was increased to 1.93-fold of the control level by 10 nM TGF-β1 treatment, while the TGF-β1-induced MMP-9 protein expressions was decreased to 81.5%, 70.4%, 55.0% of the 10 nM TGF-β1 group level by 5, 7.5 and 10μM curcumin treatment, respectively (Figure 3A, 3B). So, 10 μM curcumin has the maximal inhibitory effect. After pretreatment with 10μM curcumin...
for 30 min, the cells were treated with 10 nM TGF-β1 and 10μM curcumin for 12h, 24h, 48h, respectively. Our results showed that the TGF-β1-induced MMP-9 protein expressions were evidently suppressed by curcumin in a time-dependent way. The TGF-β1-induced MMP-9 protein expressions was decreased by 77.5%, 60.4%, 58.4% of the 10 nM TGF-β1 group level in 12h, 24h, 48h respectively. Therefore, curcumin had the best inhibitory effect at 48h. As exhibited in Figure 3 C and D, treatment with 10 nM TGF-β1 for 24h led to increase enzymatic activity of MMP-9. In contrast, curcumin dose-dependently inhibited this effect, with 10 μM curcumin showing optimum inhibitory effect.

Effect of TGF-β1 on phosphorlation of Smad2, ERK and p38MAPK in breast cancer MDA-MB-231 cells

As shown in Figure 4, 10μM TGF-β1 stimulated phosphorylation of Smad2, ERK and p38MAPK as early as 15 minutes while p-Smad2 peaked at 30 minutes. And the p-ERK and p-p38MAPK reached maximum at 60 min. The levels of total Smad2, ERK and p38 did not altered.

Effect of curcumin on activation of Smad and MAPK Pathway by TGF-β1

Cells were treated with 10μM curcumin for 0, 6, 12, and 24 h, with or without inducing of TGF-β1 for an additional 15 min. Western blot analyses indicated that TGF-β1 markedly increased activation of Smad2, ERK1/2, and p38MAPK at 12h and 24h, but did not increase levels of total Smad2, ERK1/2, p38MAPK. Curcumin at 10μM exhibited significantly inhibitive effect on TGF-β1-induced phosphorylation of Smad2, ERK1/2, p38MAPK at different intervals, and the 12 h treated group was more effective. Compared with the same time point of TGF-β1 alone treatment group, the difference was significant (P <0.001) as shown in Figure 5 A and B.

Next, cells were incubated with different concentrations (0, 5, 7.5, 10 μM) of curcumin for 12 h, followed by treatment of TGF-β1 for an additional 15 min. The results revealed that TGF-β1 at 10μM dependently induced p-Smad2, p-ERK1/2 and p-p38MAPK when compared with constant levels of total Smad2, ERK1/2, p38 MAPK. Curcumin significantly inhibited TGF-β1-induced phosphorylation of Smad2, ERK1/2 and P38MAPK in MDA-MB-231 cells at protein levels, and the inhibition effect was characterized by a concentration-dependent way, and the dose of 10 μM was regarded as the best inhibition effect (Figure 5 C, D)

Effect of PD98059, SB202580, and Curcumin on TGF-β1-induced MMP-9 in MDA-MB-231 Cells.

The MMP-9 expression and enzymatic activity were analyzed by Western blot and zymography, respectively (*P<0.05 vs. control **P<0.05 vs. TGF-β1 alone)

Discussion

About one-third of women with breast cancer develop distant metastasis and ultimately die worldwide each year (Allen et al., 2011; Nasser et al., 2012). Thus, metastatic breast cancer has been thought to be the principal challenge for the effective treatment and prevention
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In conclusion: In the present study, we explored the inhibition action of curcumin on TGF-β1–induced invasion in human breast cancer MDA-MB-231 cells and disclosed the potential mechanisms of the anti–invasion and metastasis effect. We demonstrated that (1) Curcumin dose–dependently inhibited the invasion ability induced by TGF-β1 in MDA-MB-231 cells. (2) Curcumin inhibited TGF-β1-induced MMP-9 protein expression and activity in MDA-MB-231 cells in a time- and concentration–way. (3) Curcumin time– and dose–dependently inhibited Smad2, ERK1/2, p38 MAPK phosphorylation induced by TGF-β1. (4) PD98059 and curcumin had the similar inhibitory effects on TGF-β1–induced MMP-9. Nevertheless, the lowest effect concentration of SB203580 did not affect regulation of MMP-9. These results were consistent with the results have reported by Safina A et al.. Accordingly, our findings argued that curcumin perhaps down-regulated TGF-β–induced MMP-9 via a mechanism involving ERK, Smad2 but not p38MAPK in MDA-MB-231 cells.

Moreover, previous studies have revealed that the p38 pathway might enhance cell metastasis ability by regulating actin remodeling factor HSP27 (Hedges et al., 1999) and affecting actin polymerization and cell contractility (Srinivasan et al. 2008). ERK may regulate cell motility by preventing formation of extensive actin stress fibers via suppression of tropomyosin induction by TGF-β1 or through inhibition of RhoA-Rho kinase pathway (Bakin et al., 2004; Helfman et al., 2005). So, perhaps activation of ERK, Smads and p38 MAPK signaling pathways is all required for the suppression of curcumin on TGF-β1–mediated cell migration. This speculative conclusion will also be our future research directions.

Our data revealed that the change of ERK1/2 and Smad2 phosphorylation was the key point of the anti–invasion effect of curcumin. Although our results also found that curcumin was able to reduce TGF-β1 RII expression in MDA-MB-231 cells (data does not show). However, as the lack of specific protein phosphatase inhibitors, we cannot exclude the possibility that curcumin inhibits TGF-β1–stimulated MMP-9 via directly suppressing activation of the TGF-β receptors which acts as an upstream regulator of ERK and Smad pathway.

In conclusion: In the present study, we explored the inhibition action of curcumin on TGF-β1–induced invasion in human breast cancer MDA-MB-231 cells and disclosed the potential mechanisms of the anti–invasion and metastasis effect. We demonstrated that (1) Curcumin dose–dependently inhibited the invasion ability induced by TGF-β1 in MDA-MB-231 cells. (2) Curcumin inhibited TGF-β1-induced MMP-9 protein expression and activity in MDA-MB-231 cells in a time- and concentration–way. (3) Curcumin time– and dose–dependently inhibited Smad2, ERK1/2, p38 MAPK phosphorylation induced by TGF-β1. (4) PD98059 and curcumin had the similar suppression effect on TGF-β1–induced MMP-9 protein expression and activity. All together, these findings highlight the profitable effect of curcumin, and it serves as an anti–MMP-9 factor through inhibition of the TGF-β/ Smad and TGF-β/Erk signaling pathway.
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