RESEARCH ARTICLE

Curcumin Effect on MMPs and TIMPs Genes in a Breast Cancer Cell Line

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Abstract

Curcumin (CM) possesses anti-cancer activity against a variety of tumors. Matrix metalloproteinases (MMPs) play an important role in remodeling the extracellular matrix and their activities are regulated by tissue inhibitor of metalloproteinases (TIMPs) family. Control of MMP and TIMP activity are now of great significance. In this study, the effect of CM is investigated on metastatic MMPs and anti-metastatic TIMPs genes on MDA breast cancer cells cultured in a mixture of DMEM and Ham's F12 medium and treated with different concentrations of CM (10, 20 and 40μM for various lengths of time. Reverse transcription followed by quantitative real time PCR was used to detect the gene expression levels of MMPs and TIMPs in CM-treated versus untreated cases and the data were analyzed by one-way ANOVA. At high concentrations of curcumin, TIMP-1, -2, -3 and -4 genes were up-regulated after 48 hours of treatment, their over-expression being accompanied by down-regulation of MMP-2 and MMP-9 gene expression levels in a concentration- and time-dependent manner. These results suggest that curcumin plays a role in regulating cell metastasis by inhibiting MMP-2 and MMP-9 and up-regulating TIMP1 and TIMP4 gene expression in breast cancer cells.

Keywords: Curcumin - matrix metalloproteinases - tissue inhibitor of metalloproteinases - breast cancer

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Introduction

Breast cancer is the most common leading cause of death among women worldwide (Kohrmann et al., 2009) due to distant metastases. Tumor cells produce Matrix Metalloproteinase (MMP) enzymes that destroy the basement of membranes, permitting invasion (Kohrmann et al., 2009). MMP is a family of 23 members structurally and functionally related endopeptidases (Nagase et al., 2006). MMPs are upregulated in most human tumor cell lines and their high levels are linked to metastasis (Baker et al., 2000). The stage of tumor progression is positively correlated with the expression of MMP family members (MMP-1, 2, 3, 7, 9, 11, and 14) (Overall and Lopez-Otin, 2002). MMP-2 and MMP-9 help in forming neovascularization and are involved in tumor angiogenesis mainly through their matrix-degrading capacity (John and Tuszynski, 2001). MMP-9 up-regulation was associated with a shortened relapse-free survival in breast cancer patients (Vizoso et al., 2007).

The tissue inhibitor of metalloproteinases (TIMPS) family, including TIMP-1, 2, 3, and 4, regulates the multifunctional metalloproteinase activities. TIMPs have apoptosis-inducing properties and are down-regulated in a variety of human cancer cell lines. Over-expression of TIMPs reduced the experimental metastasis of melanoma (Khokha, 1994; Montgomery et al., 1994). TIMP-1 overproduction is showed to slow carcinogenesis in transgenic mice (Martin et al., 1996; Buck et al., 1999. TIMP-2 is involved in cancer progression and metastasis (Stetler-Stevenson and Seo, 2005) and is downregulated in prostate cells and tumor samples (Pulukuri et al., 2007). Over-expression of TIMP-3 resulted in apoptosis of lung cancer cell line. In breast cancer samples expression of MMP-1,-2, -3, -9, and inhibition of TIMP-1, -2 were stronger in tumor cells than in inflammatory cells (Baker et al., 2002).

A large number of natural products have a chemo-preventive potential. Curcumin (CM) is a biphenyl compound in the herb *Curcuma longa* and possesses anti-inflammatory, anticancer, antioxidant, wound healing, and antimicrobial activities (Maheshwari et al., 2006; Sintara et al., 2012). CM has chemopreventive potential for several cancers (Huang et al., 1994; Perkins et al., 2002; Dorai et al., 2004; Choudhuri et al., 2005; Chen et al., 2006; Yang et al., 2012) and blocks steps in the carcinogenesis process. CM acts on multiple targets and inhibits activation of key cell signaling mediators, including NFκB, AP-1, Cox-2, MMP9, and EGFR (Shishodia et al., 2007). In breast cancer cells, CM's antiproliferative effects have been linked to apoptosis induction and regulates p21 expression through a p53-dependent pathway (Notoya et al., 2006; Park et al., 2002; Zheng and Chen, 2004; Choudhuri et al., 2005; Aggarwal et al., 2007; Gao et al., 2012). Despite

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a difficult in understanding the cell biological processes underlying cancer, there are few effective therapies, emphasizing the need for new insights into this disorder. Chemoprevention is a rapidly growing field in cancer research that focuses on inhibiting and delaying the onset of carcinogenesis. Studies on MMPs and TIMPS in cancer provide the basis for developing anti-metastatic cancer drugs. In this study we aimed to investigate CM effect on the metastatic property and its possible mechanism of action on MDA breast cancer cell line.

Materials and Methods

MDA-cell line (MDA-MB-231) was cultured in a mixture (1:1, v/v) of DMEM and Ham's F12 medium (Invitrogen) supplemented with 2 mmol/L L-glutamine (Invitrogen), 0.02 mmol/L nonessential amino acids (Mediatech), and 5% fetal bovine serum. Cells were treated with increasing doses of CM (Sigma, USA) 10μM, 20μM, 40μM and were incubated for various lengths of time 24-hr, 48-hr and 72-hr. The untreated cell line was act as control.

Gene expression profile

Total RNA was extracted from the CM-treated or control cell line with TRIzol (Gibco BRL), in accordance with the manufacturer's instructions. Concentrations and purity of RNA were quantified spectro-photometrically by measuring A_{260} and A_{280}; the ratio A_{260}/A_{280} of pure RNA is approximately 1.8. Complementary DNAs was synthesized with oligo-dT primers in a 20μl total volume reaction mixture using a superscript pre-amplification system (Invitrogen, USA). The expression levels of TIMPs and MMPs were detected with real-time PCR on ABI prism 7500 sequence detection system (Applied Biosystems). Real time PCR was set up and two sets of primers, as in Table 1 and conditions as previously reported (Figueira et al., 2009), were used in all reactions to yield the amplification of an endogenous control gene (GAPDH) and the specific target genes of interest. Following amplification, melting curve analysis was performed to verify the correct product according to its specific melting temperature (Tm). Experiments were performed in triplicate.

Statistical Analysis

All experiments were performed in triplicate and analyzed by one way ANOVA (Excel; Microsoft) for significant differences. P values of <0.05 were considered statistically significant. Where appropriate, the data are presented as the mean±SD.

Results

To investigate the effect of CM on the MMPs and TIMPs genes, we treated MDA human breast cancer cell line with increasing concentrations of CM and incubated for 24-hr, 48-hr and 72-hr.

The expression of TIMP1 gene in CM-treated and untreated Breast cancer MDA cells

After 24-hr incubation, there was no difference observed in the TIMP1 gene expression at the concentration of 10μM, 20μM and 40μM compared to the untreated cells. There was no difference observed in the TIMP1 gene expression at the concentration of 10μM in all days compared to the untreated cells. In the CM-treated cells with 20μM and 40μM, at 48-hr incubation, the expression of TIMP1 was significantly increased by 2.43 and 4.1 folds respectively than in the untreated cells. After 72-hr there was a significant increased in the expression level of the TIMP1 gene in the CM-treated MDA cells with 20μM and 40μM by 4.1- and 5.13 folds respectively compared to the untreated-MDA cells ($P<0.05$, Figure 1A). At higher concentrations of CM 20μM and 40μM, TIMP1 was increased after 48 hours of treatment, suggesting that CM induces anti-metastatic effect in a time- and concentration-dependent manner in MDA cells.

The expression of TIMP2 gene in MDA CM-treated and untreated Breast cancer cells

The expression of TIMP2 gene was significantly increased in CM-treated MDA cells with high concentration of 40μM. There was no significant difference observed in the TIMP2 gene expression with low concentration of CM 10μM for various lengths of time compared to the untreated cells (Figure 1B).

The expression of TIMP3 gene in MDA CM-treated and untreated Breast cancer cells

The expression of TIMP3 was increased significantly in response to treatment with 20μM and 40μM of CM after 48-hr and 72-hr incubation, whereas at 10μM the TIMP3 expression was significantly increased after 72-hr incubation compared to the untreated cells (Figure 1C).

The expression of TIMP4 gene in MDA CM-treated and untreated Breast cancer cells

There was no difference observed in the TIMP4 gene expression at the concentration of 10μM compared to the untreated cells. After 48-hr, the TIMP4 gene expression was increased significantly in response to CM-treatment with 20μM and 40μM by 2.4 and 3.3 folds respectively

Table 1. Primer Sequences

Gene	Forward primer	Reverse primer
MMP-2	5'-TTTCCATTCCGCTTCCAGGGCAC-3'	5'-TCGCACACCACATCTTTCCGTCACT-3'
MMP-9	5'-CCTGCCAGTTTCCATTCATC-3'	5'-GCCATTCACGTCGTCCTTAT-3'
TIMP1	5'-ACAACCGCAGCGAGGAGT-3'	5'-AGGTGACGGGACTGGAAGC-3'
TIMP2	5'-TTGACCCAGAGTGGAACG-3'	5'-ACCAAAGACGGGAGACGA-3'
TIMP3	5'-GTTGTAGGGTTTCTGTTGT-3'	5'-GTGTTGTCTGCTGCTTTT-3'
TIMP4	5'-TACCAGGCTCAGCATTAT-3'	5'-CCACTTGGCACTTCTTATT-3'
GAPDH	5'-AAGGATAATGGCTTACAAC-3'	5'-TCACTTAGGGCTTCTCAC-3'

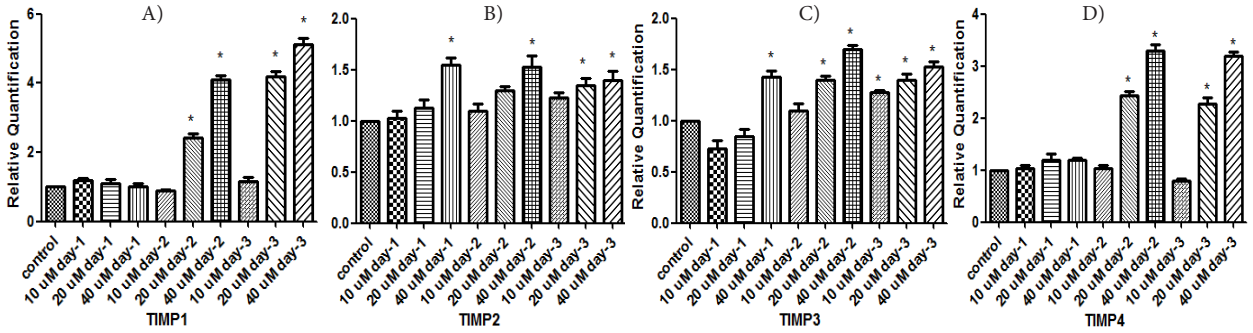

Figure 1. Showed the Expression in Breast Cancer MDA Cells and CM-treated Cells. A) TIMP1 gene expression, MDA- cells were plated in a T25 at a density 1×106 cells/flask with DMEM/F12 supplemented with 10% FBS . The cells were then treated with CM at concentrations of 10μM, 20μM, or 40μM for24-hr, 48-hr and 72-hr. For quantitative analysis, total RNA was isolated and RT followed by real time PCR was performed to investigate the gene expression level. Each bar represents the mean±S.D. calculated from three independent experiments. B) TIMP2 gene. C) TIMP3 gene. D) TIMP4 gene. Columns, mean (n = 3); *bars*, SD. *P < 0.05, statistically significantly compared with untreated control. Data are representative of at least three independent experiments with GAPDH used as the internal control.

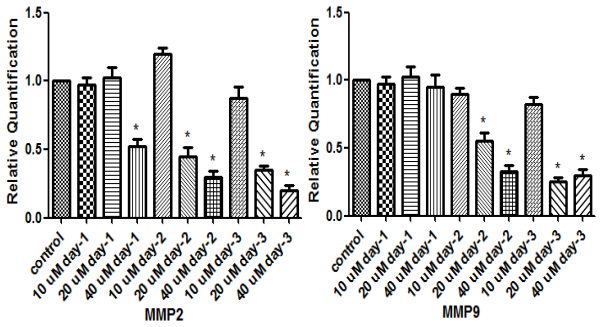

Figure 2. Showed the Expression in CM-treated and Untreated MDA Breast Cancer Cells. A) MMP2 Gene CM downregulates the MMP2 expression. MMP2 gene expression level was decreased by CM in a concentration-dependent manner (20-40μM) after 48-hr incubation. B) MMP9 gene. Columns, mean (n=3); *bars*, SD. *P <0.05, statistically significantly compared with untreated control. Data are representative of at least three independent experiments with GAPDH used as the internal control.

than in untreated cells and no further up-regulation observed after 72-hr incubations (Figure 1D).

The expression of MMP2 gene in CM-treated and untreated Breast cancer MDA cells

At the CM concentration of 10μM no difference observed in the MMP2 gene expression levels. MMP2 was reduced significantly to 0.45 and 0.35 folds in response to CM-treatment with concentrations of 20μM after 48-hr and 72-hr incubations respectively (Figure 2A). The expression of MMP2 was also reduced significantly in response to CM-treatment of 40μM to 0.3 folds after 24-hr incubation and to 0.2 folds after both 48-hr and 72-hr incubations.

The expression of MMP9 gene in CM-treated and untreated Breast cancer MDA cells

The CM concentration of 10μM causes no significant difference in the MMP9 gene expression level. The expression of MMP9 was significantly down-regulated in response to CM-treatment of 20μM and 40μM to 0.5 and 0.3 folds after 48-hr and to 0.22 and 0.3 folds respectively after 72-hr incubation (Figure 2B).

At higher concentrations of CM (20μM and 40μM) MMP2 and MMP9 were significantly decreased after 48 hours of treatment, also suggesting that CM may induce anti-metastatic effect in a time- and concentration-dependent manner in MDA cells.

Discussion

CM inhibits the proliferation of various tumor cells in culture, prevents carcinogen-induced cancers in rodents, and inhibits the growth of human tumors (Kunnumakkara et al., 2008; Lin et al., 2012). Due to its little or no toxic side effects and good bioavailability, CM possesses anticancer activities against a variety of tumors including human breast carcinoma (Nagaraju et al., 2012). MMPs function in the remodeling of the extracellular matrix that is integral for many normal and pathological processes. MMPs are up regulated and often associated with a poor prognosis for patients (Forget et al., 1999; Curran et al., 2004; Ranogajec et al., 2012). Upregulated expression of MMP-2 and -9 in tumors leads to the degradation of basement membranes (Iwasaki et al., 2002; Kato et al., 2002). There was a correlation between high expression of MMP-2 and the reduction in the survival and between the increased levels of MMP-9 with the tumor grade in breast cancer patients (Li et al., 2004). The TIMPs family, including TIMP-1, 2, 3, and 4, regulates the activity of multifunctional MMPs (Sun et al., 2010). The degradation of matrix proteins is under the control of MMPs, which in turn are regulated by their own tissue inhibitors (TIMPs). TIMPs inhibit the MMP activities and could modulate critical signaling pathways independent of metalloproteinase inhibition. TIMPs are involved in other biological processes in cancer and are decreased in some human cancer cell line (Sun et al., 2010). Control and modulation of MMP transcription and/or activation by several naturally occurring substances is now representing novel options for the control of MMP and TIMP activity. In this study, we demonstrate the CM effects on the metastatic and anti-metastatic mechanism of action in breast cancer cell line.

There was a correlation between high expression levels of MMP-2 and -9 and a higher rate of distant metastases

(Vizoso et al., 2007).The expression of MMP-2, -9 genes was identified in breast cancer tissue (Decock et al., 2007) and with high expression levels in comparison to normal breast tissue (Pacheco et al., 1998). In addition, higher MMP-9 protein level was detected in breast cancer tissue when compared to normal breast tissue (Przybylowska et al., 2006). In this study we found that at the CM concentration of 10μM no difference observed in MMP2 and MMP9 genes expression levels. MMP2 was reduced significantly in response to CM-treatment with concentrations of 20μM after 48-hr and 72-hr incubations. The expression of MMP2, was also reduced significantly in response to CM-treatment of 40μM to 0.53 folds after 24-hr incubation and to 0.3 and 0.2 folds after 48-hr and 72-hr respectively incubations.

The expression of MMP9 was significantly down-regulated in response to CM-treatment of 20μM and 40μM to 0.5 and 0.3 folds after 48-hr and to 0.22 and 0.3 folds respectively after 72-hr incubation. At higher concentrations of CM (20μM and 40μM) MMP2 and MMP9 were significantly decreased after 48 hours of treatment, also suggesting that CM may induce anti-metastatic effect in a time- and concentration-dependent manner in MDA cells. CM suppresses MMP expression which is played a major role in mediating neovascularization and is increased during tumor progression (Park and Conteas, 2010). Our results, combined with previous studies, suggest that CM reduced the metastasis of cancer cells (Park and Conteas, 2010). Consistent with our data, CM down regulates MMP-9 expression but this downregulation occurred by inhibiting NF-κB and AP-1 binding to the DNA promoter region (Woo et al., 2005). Another study showed that CM reduced invasion by inducing osteopontin (Philip et al., 2004) or caused significant inhibition of tumor necrosis factor α and increased the vascular cell adhesion molecules-1 (VCAM-1) expression, of the NF-κB pathway (Lee et al., 2006). The observed efficient reduction of MMP-2 and -9 gene expression levels during the CM treatment of MDA breast cancer cells with different concentration suggesting that CM can suppress breast cancer metastasis.

Overexpression of TIMPs reduced experimental metastasis of melanoma (Khokha, 1994; Montgomery et al., 1994). TIMP-1 overproduction slowed chemical carcinogenesis in skin and liver carcinogenesis in transgenic mice (Martin et al., 1996; Buck et al., 1999; Bica et al., 2010). TIMPs, aside from inhibition MMP (Valente et al., 1998), also could suppress receptor tyrosine kinase signaling independent of metalloproteinase inhibition (Stetler-Stevenson, 2008). In this study we have presented data demonstrating that after 24-hr incubation no difference observed in the TIMP1 gene expression at different concentration. There was no difference observed in the TIMP1 gene expression at the concentration of 10μM in all days compared to the untreated cells. In the CM-treated cells with 20μM and 40μM, at 48-hr incubation, the expression of TIMP1 was significantly increased by 2.43 and 4.1 folds and after 72-hr was significantly increased by 4.2- and 5.1 folds. At higher concentrations, 20μM and 40μM, CM induces anti-metastatic effect in a time- and concentration-dependent manner in MDA cells. It was well known that TIMP-1 has inhibitory activity against MMP-9 (Figueira et al., 2009). Our study showed that the increase of the TIMP-1 mRNA level in breast cancer cell line during CM treatment was associated with the down-regulation of MMP-9. This suggests CM may inhibit MMP9 production, rather than decrease of its synthesis, leading to inhibition the degradation of ECM. Thus, it appears that CM significantly reduces the functional ability of MMP-9 by both decreasing the rate of production as well as increasing its natural inhibitor, TIMP-1. Administration of CM can return the relationship between MMPs and TIMPs to their normal concentration. CM controls cancer progression by either blocking tumor growth or inhibiting its invasive and aggressive potential.

TIMP-2 is involved in cancer progression and metastasis. High TIMP-2 concentration inhibits the proMMP-2 activation (Munshi et al., 2004). Study using breast cancer samples demonstrates that the inhibition of TIMP-1 and TIMP-2 were stronger in tumor cells than in inflammatory cells within the tumor section (Sun, 2010). In this study there was significantly increased in CM-treated MDA cells with high concentration of 40μM compared to the untreated cells (Figure 1B). TIMP-2 is normally expressed in breast stromal tissue; however, increased expression has been found in ductal carcinoma *in situ* and in invasive breast carcinomas (Kim et al., 2006; Kohrmann et al., 2009) TIMP-2 has been found to stimulate cell growth and inhibit apoptosis in breast cancer cells, as well as to inhibit endothelial cell growth and abrogate angiogenesis (Chirco et al., 2006). Increased expression of TIMP-2 in breast cancer tissue has also been associated with tumor recurrence and development of metastasis (Zhang et al., 2007)

TIMP-3 has been found to induce apoptosis in both normal and malignant cells and also to inhibit endothelial cell motility and proliferation (Mannello et al., 2005) In addition to inhibiting tumor growth, TIMP-3 has also been found to be a potent inhibitor of angiogenesis (Qi et al., 2003). Overexpression of TIMP-3 resulted in apoptosis of lung cancer cells. Adenoviral delivery of TIMP-3 gene inhibited the growth of tumors in nude mice, and was associated with a greater therapeutic effect than either TIMP-1 or -2 gene delivery (Finan et al., 2006). In this study, the expression of TIMP3, was found to be increased significantly in response to treatment with 10, 20, 40 μM of crurcumin, followed by slightly increasing in its expression in the followed days. TIMP-3 is a cell-cycle-regulated gene that is normally found in the breast epithelium; reduced TIMP-3 expression in breast tumor and peri-tumoral tissues has been linked to cell cycle deregulation and tumor cell proliferation (Mylona et al., 2006). Reduced expression of TIMP-3 in breast cancer tissue has been associated with poor disease-free survival (Kotzsch et al., 2005). Down regulation of TIMP3 can cause increase in MMP2.

The expression of TIMP4, was increased significantly in response to treatment with 20 μM and 40 μM by 2.4 and 3.3 folds respectively after 48-hr and 72-hr incubations than in untreated cells. There was no difference observed in the TIMP4 gene expression at the concentration of 10μM compared to the untreated cells. According to our

results, CM increases the expression of TIMP4 after 72-hr incubations of treatment and decreases the expression level of MMP9 and MMP2. To our knowledge, currently there are no data available for the effect of CM on the TIMPs regarding their expression in breast cancer cell in literature. Overall, this study on MMPs and TIMPS in cancer provides the rationale for developing cancer drugs that target TIMP and MMP activities. Curcumin plays an important role in regulating MDA cell metastasis by inhibiting the expression of MMP-2 and MMP-9 and increasing the expression of TIMP1 and TIMP4 in breast cancer cells. Therefore it can help in tailoring new anti-metastatic cancer therapy.

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