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Curcumin inhibits metastatic progression of breast cancer cell through suppression of urokinase-type plasminogen activator by NF-kappa B signaling pathways

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Abstract Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is extracted from the plant *Curcuma longa*. It was recently reported for its anticancer effect on several types of cancer cells in vitro however, the molecular mechanisms of this anticancer effect are not fully understood. In the present study, we evaluated the effects of curcumin on human mammary epithelial carcinoma MCF-7 cells. Cells were treated with curcumin and examined for cell viability by MTT assay. The cells invasion was demonstrated by transwell assay. The binding activity of NF-κB to DNA was examined in nuclear extracts using Trans-AM NF-κB ELISA kit. Western blot was performed to detect the effect of curcumin on the expression of uPA. Our results showed that curcumin dose-dependently inhibited ($P < 0.05$) the proliferation of MCF-7 cells. Meanwhile, the adhesion and invasion ability of MCF-7 cells were sharply inhibited when treated with different concentrations of curcumin. Curcumin also significantly decreased ($P < 0.05$) the expression of uPA and NF-κB DNA binding activity, respectively. It is concluded that curcumin inhibits the adhesion and invasion of MCF-7 cells through down-regulating the protein expression of uPA via of NF-κB activation. Accordingly, the therapeutic potential of curcumin for breast cancer deserves further study.

Hong Zong and Feng Wang contributed equally to this work.

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Introduction

Breast cancer is the most common malignant tumors and the second highest mortality among women [1]. Survival rates for breast cancer have greatly increased with significant improvement in surgical technology and therapy regimens over the last three decades, especially for early-stage breast cancer. However, there are currently no effective treatments for metastatic breast cancer [2, 3]. As one of the key events in tumor metastasis and malignant transformation, cell invasion is frequently accompanied by enhanced mobility of tumor cells. Obtaining a better understanding of the factors and mechanisms that regulate breast cancer cell invasion is crucial to the development of treatments that limit breast cancer metastasis.

Curcumin (diferuloylmethane), a bioactive component derived from the roots of the East Indian plant *Curcuma longa*, has been widely used in therapeutic preparations for centuries owing to its anti-inflammatory, antioxidant, and chemotherapeutic properties [4, 5]. Especially for its anti-carcinogenic property, still has been the subject of a great deal of interest. Increasing evidence indicated that curcumin has anticancer effects against different types of human tumor cells, including of ovarian cancer cells [6], colon cancer cells [7] and astroglioma cells [8]. This anticancer effects of curcumin were identified through interfering with the cell cycle, inducing apoptosis, and inhibiting the invasive potential of cancers [9, 10]. However, the underlying molecular mechanisms of this anticancer effects are still under investigation, especially for its anti- metastatic potential in breast cancer.

Metastasis is one of the major causes of mortality in cancer patients. The initial step in metastasis is local invasion and occurs as a coordinated multistep process involving in the adherence and migratory properties of the primary cancer cells and extensive degradation of the extracellular matrix (ECM) surrounding the primary tumor cells [11]. A number of proteinases are involved in the degradation of ECM by cancer cells, including of serine proteinase, matrix metalloproteinases (MMPs), and cathepsins [12]. Urokinase-type plasminogen activator (uPA), as a member of the serine protease that interacts with the uPA receptor (uPAR) and induces the conversion of inert zymogen plasminogen to protease plasmin, further degrades surrounding matrix components such as fibronectin, type IV collagen, and laminin, finally allows cancer cells to migrate to the distant sites [13]. It is well demonstrated that uPA plays a significant role in tumor growth and metastasis [14, 15]. NF-κB-responsive element is present in the promoter region of uPA, which mediates uPA secretion and regulates cell migration and ECM invasion in cancer metastasis. Curcumin's effects on the NF-κB pathway have been studied in multiple human carcinomas [16, 17]. However, whether curcumin was involved in the regulation of breast cancer cell invasion through controlling uPA secretion via NF-κB signaling pathways is not well documented.

In the present study, we used human breast cancer cell line MCF-7 to investigate the effects of curcumin on the breast cancer cell adhesion and invasion during metastasis process. Our finding demonstrated that curcumin inhibits the adhesion and invasion of breast cancer cells by suppression of the activation of NF-κB. These lead to the reduction of uPA secretion, and all of these ultimately control the cell motility, invasiveness, and metastatic spread of breast cancer.

Materials and methods

Materials

Curcumin (diferuloylmethane) with a purity of >98% was purchased from Sigma-Aldrich Corporation (St. Louis, MO), and it was dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution and stored at −20°C. Primary antibodies for uPA and GAPDH were purchased from Cell Signaling Technology (Danvers, MA).

Cell culture

Human mammary epithelial carcinoma cells (MCF-7) were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum(FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM L-glutamine. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay was performed to evaluate cytotoxicity of curcumin in MCF-7 cells. Briefly, cells were seeded at a density of 5000 cells/well on a 96-well plate. After attachment, various concentrations of curcumin (0, 10, 20, 50, 100 μM) were added for 48 h. Then the cells were washed with PBS and 200 μl MTT (0.5 mg/ml) was added to each well and incubated until formazan was constituted. After medium was removed, formazan was dissolved with DMSO and then measured using microplate reader (Bio-TEK Instrument, Winooski, VT) at 570 nm. Six wells were used for each treatment concentration, and the experiment was repeated three times.

Adhesion assay

Adhesion assay was performed as previously described. In brief, 96-well plate was coated with 50 μM Matrigel (100 μg/ml) and incubated overnight at 4°C. Plate was then blocked with 0.1% bovine serum albumin for 2 h at room temperature followed by washing three times with phosphate buffered saline (PBS). MCF-7 cells were preincubated with curcumin (0–100 μM) for 20 min at 37°C. Cells (10,000 cells/well) were added to each well in triplicate and incubated for 1 h at 37°C. Plates were then washed three times with PBS to remove unbounded cells. The crystal violet assay performed to calculate the number of attached cells. Wells containing known cell numbers (0, 1000, 2000, 5000, 10000, 20000, or 40000 cells/well) were treated in a similar fashion to establish standard curves. The optical density was measured using microplate reader at 570 nm. The experiment was repeated four times.

Invasion assay

The Boyden chamber (BD Biosciences, San Jose, CA) was used to evaluate the cell invasion of MCF-7 cells as described. Cells were pre-cultured in serum-free medium in the absence or presence of curcumin for 24 h. Cells (8×10^4) suspended in 0.5 ml serum-free medium with or without curcumin were applied onto the upper compartment of invasion chamber coated with Matrigel (30 mg/filter). The lower compartment was filled with complete medium in the absence or presence of curcumin. The chamber was incubated at 37°C for 12 h and the filters

were removed. Cells invaded on the bottom of inserts were fixed, stained, and counted under a microscope. Each experiment was performed in triplicates.

Preparation of nuclear extracts and NFκB activation measurement

The binding activity of NFκB to DNA was examined in nuclear extracts using Trans-AM NF-κB ELISA kit (Active Motif, Carlsbad, CA). Briefly, cells were lysed in a hypotonic solution (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2 and 0.1% Triton X-100, protease inhibitor cocktail) on ice for 15 min and centrifuged at 13,000 rpm to pellet nuclei. Then the pellet was re-suspended in nuclear extract buffer on ice for 15 min. The lysates were centrifuged at 13,000 rpm, and supernatants containing the nuclear proteins were collected. Protein concentrations were determined using the method of Bradford (Bio-Rad, Hercules, CA). NF-κB activation was measured according to the manufacturer's recommendations. Following color development, absorbance was read at 450 nm within 5 min. Each experiment was performed in triplicates.

Western immunoblot analysis

Western blot analysis was carried out as following described. Whole cell extracts were prepared by using protein lysis buffer (50 mM HEPES, pH 7.4, 0.5 M NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 4 mM sodium pyrophosphate, 10 mM sodium fluoride, 1% Triton X-100, and protease inhibitors cocktail). After centrifugation at $13000\times g$ for 30 min, the supernatants were collected. Protein concentrations were determined by Bradford assay. The protein samples were separated on 10% SDS-PAGE gels, and electrically transferred to PVDF membranes. The membranes were probed with anti-uPA and anti-GAPDH antibodies (Cell Signaling Technology, Beverly, MA). The immunoreactive signals were visualized using ECL reagents and analyzed by densitometry. Changes in uPA and GAPDH were quantified by scanning densitometry (model GS 670; Bio-Rad, Hercules, CA). Data on uPA expression was normalized to GAPDH.

Statistics procedures

All data were expressed as means ± SEM of at least three independent experiments. The statistically significant differences compared with untreated group were analyzed using one-way ANOVA or Student's *t* test. $P < 0.05$ were considered to be statistically significant.

Results

Effects of curcumin on the viability of MCF-7 cells

To examine the effects of curcumin on MCF-7 cell viability, MTT assay was performed. MCF-7 cells were plated onto 96-well plate and treated with different concentrations of curcumin (0–100 μM) for 48 h. As shown in Fig. 1, curcumin dose-dependently ($P < 0.05$) inhibited cell viability. These inhibitory effects were observed after incubation with 20, 50, and 100 μM curcumin, reducing cell growth by 37, 54, and 73%, respectively.

Effects of curcumin on the adhesion of MCF-7 cells

Given the initial step of tumor metastasis is degradation of the ECM surrounding the primary tumor cells, we performed the adhesion assay to evaluate the effect of curcumin on ECM of tumor cells. MCF-7 cells with different concentrations of curcumin treatment were added to Matrigel coated plates. Our results showed that curcumin significantly inhibited the adhesion of MCF-7 cells in a dose-dependent manner, compared with untreated control (Fig. 2).

Effects of curcumin on the invasion of MCF-7 cells

To investigate the effects of curcumin on the invasion of MCF-7 cells, cells that invaded through Matrigel coated polycarbonate filter in the Boyden chamber were analyzed. After 12 h incubation, cells showed significantly ($P < 0.05$) decreased invasiveness respond to curcumin

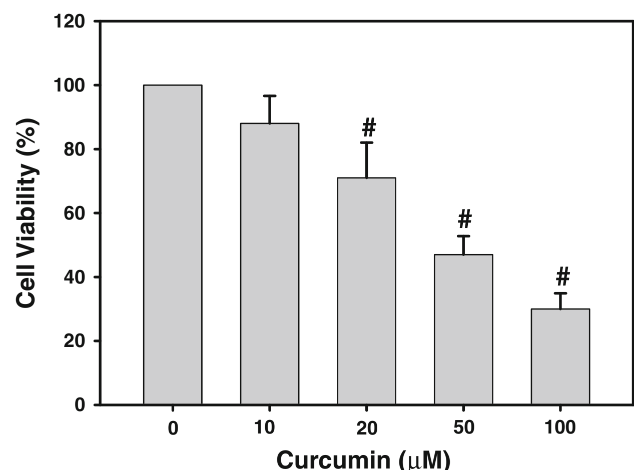

Fig. 1 Cell viability responds to curcumin treatment. Cells were treated with different concentrations of curcumin (0–100 μM) for 48 h. Cell viability was determined with MTT assay, and the graph showed the results of three independent experiments. The results are represented as a percentage of absorbance relative to control cells (100%). #Differ ($P < 0.05$) from the control

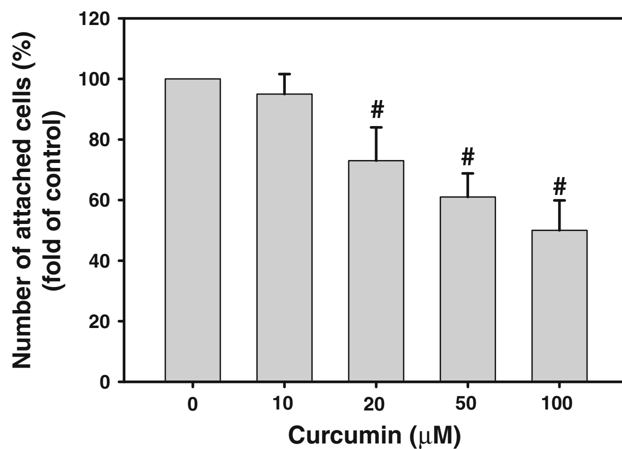

Fig. 2 Effects of curcumin on cell adhesion. Cells were preincubated with curcumin (0–100 μM) for 20 min and then seeded into wells. After attachment for 1 h, cells were washed three times with PBS to remove unbounded cells. The crystal violet assay was performed to calculate the number of attached cells. Quantified data are expressed as means ± SEM from four independent experiments. #Differ ($P < 0.05$) from the control

treatment (Fig. 3). These results demonstrated that curcumin markedly suppressed the invasion of breast cancer cells.

Effects of curcumin on the expression of uPA and the DNA binding activity of NF-kB in MCF-7cells

It is well known that uPA is deeply involved in the tumor metastatic process. To test whether curcumin affects the expression of uPA, Western blot analyses were employed. The protein levels of uPA was dose-dependently decreased by curcumin in MCF-7 cells (Fig. 3b). Moreover, the impaction of curcumin on the NF-κB pathway have been observed in multiple human carcinomas. Thus, the DNA binding activity of NF-κB were analyzed in this study. As shown in Fig. 3, curcumin significantly ($P < 0.05$) decreased NF-κB activation in a dose-dependent manner (Figs. 4 and 5).

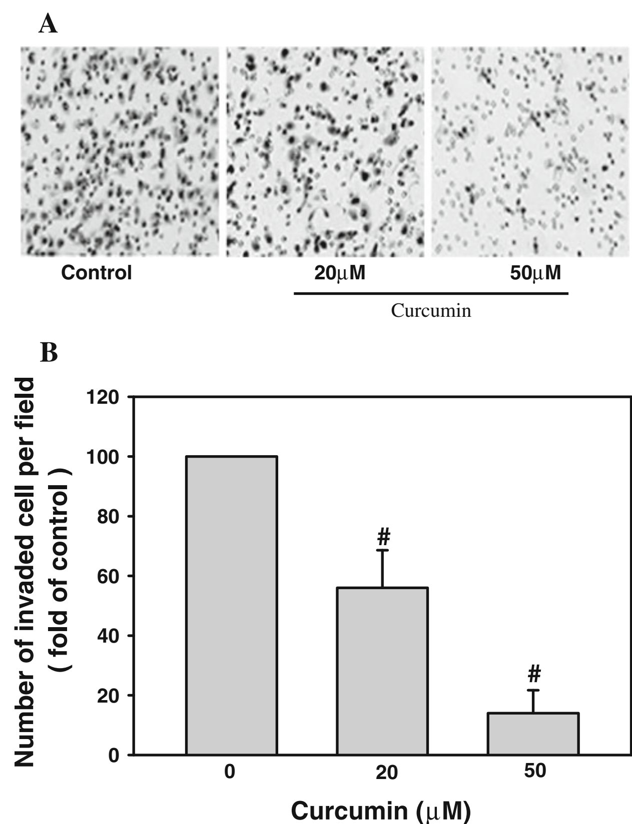

Fig. 3 Effects of curcumin on cell invasion by transwell assay. Cells suspended in serum-free DMEM were overlaid in the upper chamber of each transwell. After incubation with curcumin (0, 20, and 50 μM) for 12 h, invaded cells were stained with crystal violet and recorded under a microscope mounted with a CCD camera. **a** photographs are depicted invasion of MCF-7 cells. **b** Quantified data are expressed as means ± SEM from three independent experiments. #Differ ($P < 0.05$) from their control

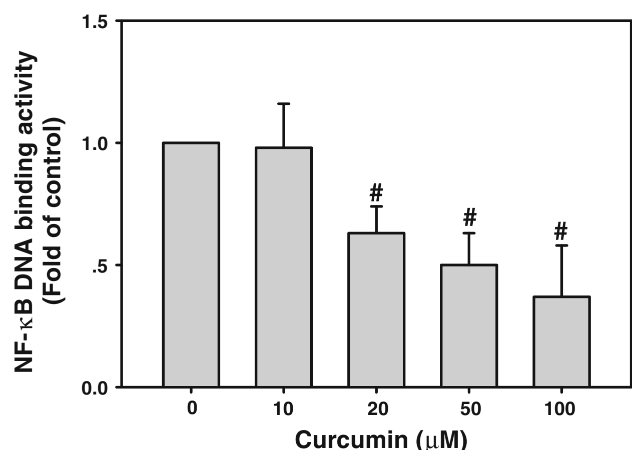

Fig. 4 Effects of curcumin on NF-κB activation Cells were pretreated with different concentrations of curcumin (0–100 μM) for 24 h. The nuclear extracts were prepared and NF-κB activation was measured. Quantified data are expressed as means ± SEM. Each experiment was performed in triplicates. #Differ ($P < 0.05$) from the control

Discussion

Curcumin is one of the most widely characterized of the phytochemicals and is the active ingredient in the rhizome of the plant turmeric, and has both antioxidant and anti-inflammatory properties. Curcumin has been shown to protect against carcinogenesis and prevent tumor formation and development in several types of cancer [4, 5, 9]. In the present study, we provided evidences that curcumin was able to inhibit adhesion and invasion of human breast cancer cells through NF-κB-dependent downregulation of

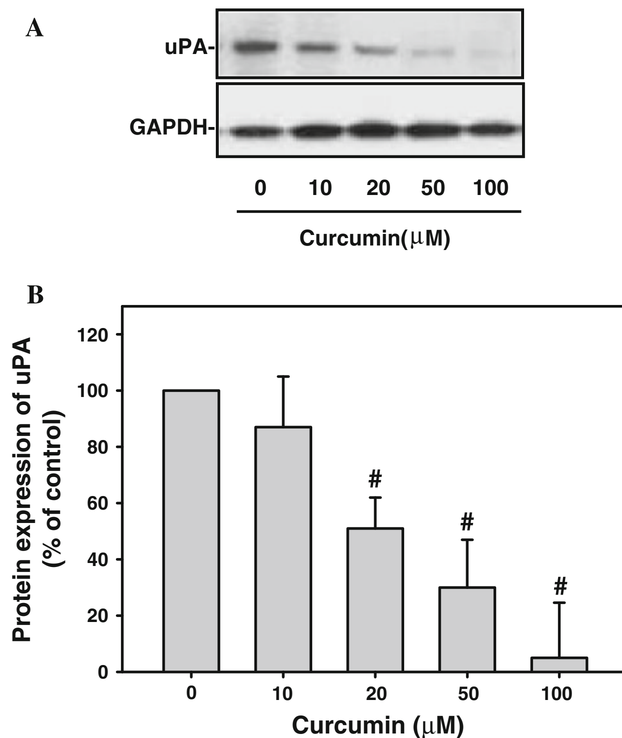

Fig. 5 Effects of curcumin on uPA protein expression. Cells were cultured in culture dishes until reaching 60–70% confluence. Then cells were treated with curcumin (0–100 μM) for 24 h. Proteins were subjected to Western blot analysis and detected using antibody against uPA and GAPDH. Data normalized to GAPDH are expressed as means ± SEM fold of the control from three individual experiments. #Differ ($P < 0.05$) from their control

uPA expression, suggesting that curcumin might possess anti-metastatic potential in human breast cancer.

Increasing evidences have indicated that curcumin is able to inhibit the growth of different types of cancer cells including ovarian [6], gastric [18], and neuroblastoma cells [19] in culture. This inhibitory effect was also verified in this study. Our data showed that curcumin significantly inhibited breast cancer cell growth, especially at high dose concentration. However, little is known about the functional role of curcumin in breast cancer metastatic progression.

Cancer metastasis is a highly coordinated multistep process involving cell invasion, cell–cell and cell–matrix adhesion, and remodelling of the ECM. Many studies indicate that a number of proteinases are involved in the degradation of the ECM by cancer cells, including of MMPs, serine proteinase, particularly uPA-plasmin system [20]. Comprising several interdependent components, the uPA-system has a number of distinct but complementary functions in this tightly regulated multi-step process. The secretion of uPA is crucial in cancer cell metastasis, and deeply involved in cancer cell adhesion and invasion [21–23]. Our date demonstrated that curcumin significantly decreased the expression of uPA in a dose-dependent manner, which further result in the inhibitory effect of breast cancer cell adhesion and invasion respond to curcumin treatment.

uPA is a member of the serine protease family, which induces the conversion of the inactive zymogen plasminogen to the active proteinase plasmin. Independent of proteolysis, uPA enhances cell invasion through activation of several migration-associated signalling molecules such as extracellular signal-regulated kinases, focal adhesion kinases and signal transducers and activators of transcription 1 (STAT1) [24, 25]. However, the signaling pathways by which curcumin controls uPA secretion through activation of NF-κB in breast cancer cells are not well defined. Curcumin's effects on the NF-κB pathway have been studied in multiple human carcinomas. After NF-κB activated, the released NF-κB translocates to the nucleus where it acts as a transcription factor. Genes regulated by NF-κB are involved in immune and inflammatory responses, and in the control of cell proliferation, apoptosis, metastasis and angiogenesis [26–28]. Couple of studies demonstrated that curcumin is an inhibitor of NF-κB, this is in agreement with our current data that curcumin dose-dependently suppressed the activation of NF-κB. Therefore, the anti-metastatic effects of curcumin in human breast cancer may be mediated by inhibiton of the NF-κB signaling pathway.

In summary, this preliminary investigation has shown that the anti-metastatic effect of curcumin may be mediated by the decrease of uPA expression. Moreover, curcumin dose-dependently suppress the activation of NF-κB, this effect may be attributed to a dose-dependent decrease in uPA protein levels. These findings reveal a new therapeutic potential for curcumin on anti-metastatic therapy.

Conflict of interest The authors declare that they have no competing interests.

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