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Curcumin inhibits lung cancer cell migration and invasion through Rac1-dependent signaling pathway

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Abstract

Curcumin, a natural and crystalline compound isolated from the plant *Curcuma longa* with low toxicity in normal cells, has been shown to protect against carcinogenesis and prevent tumor development. However, little is known about antimetastasis effects and mechanism of curcumin in lung cancer. Rac1 is an important small Rho GTPases family protein and has been widely implicated in cytoskeleton rearrangements and cancer cell migration, invasion and metastasis. In this study, we examined the influence of curcumin on *in vitro* invasiveness of human lung cancer cells and the expressions of Rac1. The results indicate that curcumin at 10 μM slightly reduced the proliferation of 801D lung cancer cells but showed an obvious inhibitory effect on epidermal growth factor or transforming growth factor β1-induced lung cancer cell migration and invasion. Meanwhile, we demonstrated that the suppression of invasiveness correlated with inhibition of Rac1/PAK1 signaling pathways and matrix metalloproteinase (MMP) 2 and 9 protein expression by combining curcumin treatment with the methods of *Rac1* gene silence and overexpression in lung cancer cells. Laser confocal microscope also showed that Rac1-regulated actin cytoskeleton rearrangement may be involved in anti-invasion effect of curcumin on lung cancer cell. At last, through xenograft experiments, we confirmed the connection between Rac1 and the growth and metastasis inhibitory effect of curcumin *in vivo*. In summary, these data demonstrated that low-toxic levels of curcumin could efficiently inhibit migration and invasion of lung cancer cells through inhibition of Rac1/PAK1 signaling pathway and MMP-2 and MMP-9 expression, which provided a novel insight into the molecular mechanism of curcumin against lung cancer.

Keywords: Curcumin; Migration; Invasion; Rac1; Actin cytoskeleton; MMP-2/9

1. Introduction

Lung cancer, the leading cause of cancer deaths, has the most rapidly increasing incidence rate in the developed country and in China. Clinical data showed that most lung cancer patients eventually suffered from relapse and/or metastasis after complete excision of the cancer, even if they were at stage IA [1]. Despite great progresses have been made in the last decades, the detailed mechanism of lung cancer relapse and metastasis is not fully understood.

Rac1, an important small Rho GTPases family protein, has been widely implicated in cytoskeleton rearrangements and cancer cell migration, invasion and metastasis [2]. Overexpression of Rac1 is considered as an independent predictor of adverse outcome of some carcinomas [3]. Inhibition of Rac1 expression or disruption of its function significantly reduces cancer cells metastasis in many tumor models [4,5]. Our recent studies also showed that overexpression of Rac1 was widespread in primary lung cancer patients. Silence of Rac1 expression by shRNA suppressed lung cancer cells migration, invasion and induced rearrangements of the actin cytoskeleton in lung cancer cells [6]. Rac1 regulates cellular functions such as cytoskeletal dynamics, cell adhesion and transcription via activating PAK1 (one of the best characterized member of Rac1 effectors) and other downstream signaling molecules. Therefore, agents that inhibiting Rac1 or its downstream targets might have anticancer metastatic effect.

Curcumin (diferuloylmethane), an active component of the spice turmeric (*Curcuma longa*), has chemopreventive and therapeutic properties against many tumors both *in vitro* and *in vivo* [7,8]. Several studies have shown that curcumin induces apoptosis more potently in cancer cells than in normal cells and attributed its inhibitory effect to the inhibition of angiogenesis nitric oxide synthase, receptor tyrosine kinase and protein kinase C activities and regulation of certain gene transcriptional factors, such as c-Jun/AP-1, JNK, Nuclear factor κB

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(NF-κB) and P53 [8–11]. Previous studies from our lab showed that curcumin inhibited cell proliferation and induced cell apoptosis in lung cancer through the modulation of lysosomal pathway and reactive oxygen species-dependent mitochondrial signaling pathway [7,12]. Recently, some studies on the anticancer effect of curcumin have focused on anti-invasion and antimetastasis aspect [13,14]. However, the effects of curcumin on lung cancer metastasis and underlying molecular mechanism remains remain largely unknown. The Rac1 signaling pathway is closely associated with tumor invasion and metastasis, and curcumin showed a potent inhibitive role on metastasis in various cancer cells, which implies an inner relationship between inhibitory effects of curcumin and Rac1 signaling.

In present study, we determined the effects of curcumin on the migration and invasion of lung cancer cells both *in vitro* and *in vivo* systems and further demonstrated that the inhibitory effects of curcumin were relate to the inhibition of Rac1/PAK1 signaling pathways, matrix metalloproteinase (MMP) 2/9 expression and actin cytoskeleton rearrangements. These results provide a novel insight into the molecular mechanisms of curcumin in inhibition of lung cancer cell migration and invasion, and also show potential therapeutic value of curcumin in preventing lung cancer metastasis.

2. Materials and methods

2.1. Reagents and cell culture

Curcumin was purchased from Sigma Chemical Co. Rac1 antibody was purchased from Upstate Biotechnology Inc (New York, NY, USA). PAK1, p-PAK1, MMP-2 and MMP-9 antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Human epidermal growth factor (EGF) and transforming growth factor β1 (TGF-β1) were obtained from (PreproTech GmbH, Hamburg, Germany). A Rac1L61 plasmid, encoding active form of Rac1, was provided by Dr. Liang Fan (Nanfang Hospital affiliated to Southern Medical University, China). Plasmid expressing green fluorescent protein (GFP)-tagged negative shRNA or Rac1 shRNA was generated as described previously [6].

Human large cell lung carcinoma 801D cell line was purchased from the Cell Bank at the Chinese Academy of Sciences, which was first developed by Dr. Lezhen Chen [15], The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

2.2. Real-time cell analysis cytotoxicity testing

Real-time cell analysis (RTCA) system (Roche Applied Science, Indianapolis, IN, USA) was employed for dynamic assessment of curcumin toxicity. The principle and use of the RTCA system have been described previously [16]. Briefly, under the control of RTCA software, the sensor analyzer automatically selected wells to be measured and continuously monitored changes in electrode impedance of cultivated cells in the electronic sensor plate. Cell index (CI) is used to represent cell status based on electrical impedance, which is proportional to the culture area covered by attached cells so that toxicity-induced cell shrinkage and detachment of cells from the plate due to cell death result in a lower CI value. In the study, the effect of the curcumin was monitored dynamically for every 15 min. The CI against the time was plotted.

2.3. Assessment of cell proliferation by MTT assay

The antiproliferative effect of curcumin on 801D lung cancer cells was also determined by the 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye uptake method. Briefly, the cells were seeded in quintuplicate at a density of 5×10^3 cells per well in a 96-well plate in a final volume of 0.2 ml medium and incubated for 24 h at 37°C. Curcumin was added to each well and incubated for another 24 h. The cells were washed with phosphate-buffered saline (PBS), and 20 μl MTT solution (5 mg/ml) was added to each well. After a 4-h incubation at 37°C, 150 μl isopropanol was added. The absorbance of MTT formazan was measured at 490 nm.

2.4. Cell transfection

Cells plated in 6-well plates were transfected with indicated plasmids using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, GFP positive cells were sorted using fluorescence activated cell sortor (FACS) and used for different experiments.

2.5. Cell migration assay

Cell migration was assayed by wound healing assay as described previously [9]. Initially, cells were allowed to grow to 80%–90% confluence in 12-well plates precoated with 0.1% gelatin (Sigma) and then starved with serum-free RPMI 1640 medium overnight. After that, cells were scraped by pipette tips to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and the cells were exposed to indicated concentrations of EGF or TGF-β1. The wound closure was monitored and photographed at 0 and 24 h under a Leica inverted microscope. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end of the incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Migrated cells across the white lines were counted in six random fields from each triplicate treatment, and data are presented as mean±S.D.

2.6. Cell invasion assay

Invasion assay was carried out using modified matrigel Boyden chambers consisting of 24-well Millicell (Millipore Corporation, Shanghai, China) membrane filter (8-μm pore size) as described previously [13]. Dilute Matrigel (50 μl; Becton-Dickinson; 1:3) in serum-free cold cell culture media and applied to the top side of filter. Briefly, cells were trypsinized and resuspended in serum-free medium. Two hundred microliters of the cell suspension (10^5 cells) with 10 μM curcumin was added to the upper chamber of each well and 0.1% of dimethyl sulfoxide (DMSO) as the solvent control. The bottom chambers were filled with 500 μl RPMI 1640 medium supplemented with 10% fetal bovine serum and growth factor (EGF or TGF-β1). The chamber was incubated for 20 h at 37°C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab. Cells invading across the matrigel to the lower surface of the membrane were fixed with methanol and stained with 0.5% crystal violet. The invading cells on the lower surface of the membrane filter were counted with a light microscope. The data presented are the average number of cells attached to the bottom surface from five random fields. Each experiment was carried out in triplicate.

2.7. Laser confocal microscope

To determine the effect of curcumin on cell morphology and cytoskeleton, cells were plated in six-well plates containing 12-mm glass coverslips and grown for 16 h and then treated with curcumin and indicated concentrations of growth factor (EGF or TGF-β1). After the exposure period, the medium was removed, and the cells were washed with PBS and then fixed with 4% formaldehyde dissolved in PBS for 10 min at room temperature and permeabilized for 10 min with 0.2% TritonX-100. Cells were incubated with 500 ng/ml tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma) at room temperature in PBS containing 0.1% Triton X-100 for 45 min. The cells were examined and photographed by confocal microscopy.

2.8. Reverse transcription polymerase chain reaction assay

Total RNA was isolated from each group of cells using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Obtained complementary DNAs were amplified using specific primers. The polymerase chain reaction (PCR) from each sample was performed by the following conditions: 5 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 55°C, and final extension for 1 min at 72°C. Primers for Rac1 are as follows: 5′-ATGCAGGCCATCAAGTGTGTGGTG-3′ (sense) and 5′-TTACAACAGCAGG-CATTTTCTCTTCC-3′ (antisense).

2.9. Western blot analysis

The whole-cell extracts were prepared in RIPA buffer [20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 40 mM NaF, 10 mM $Na_4P_2O_7$ and 1 mM phenylmethanesulfonylfluoride (PMSF)]. Thirty micrograms of cellular protein of each sample was applied to immunoblot following 10% SDS-polyacrylamide gel electrophoresis and probed with specific antibodies as indicated, followed by a horseradish peroxidase-conjugated goat antimouse or antirabbit antibody (Millipore). Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore) according to the manufacturer's instructions. Quantification of reactive protein bands was performed by densitometric analysis, and the fold change was calculated by normalizing with control β-actin levels.

2.10. In vivo animal studies

The cell viability and cell number of 801D cells were calculated by trypan blue. Then, 1×10^7 live cells in RPMI-1640 medium were injected subcutaneously into the flanks of mice (nude mice, average 25 g, purchased from Shanghai Laboratory Animal Center, Shanghai, China).

All experimental procedures used in this study had been approved by the ethics committee in the 117th Hospital of PLA, and all animal experiments had been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The authors who performed experiments had given their informed consent