The anti-cancer efficacy of curcumin scrutinized through core signaling pathways in glioblastoma

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Received March 16, 2010; Accepted April 29, 2010

DOI: 10.3892/ijmm_00000455

Abstract. Curcumin has been verified as an anti-cancer compound via multiple molecular targets. Its effective mechanisms include cell cycle arrest, inducing apoptosis, suppressing oncogenes, and enhancing tumor suppressor genes. The resistance of cells to chemotherapy, however, derives from the variable genetic aberration of cancer cells. Consequently, the core signaling pathways of glioblastoma have been explored to evaluate the efficacy of curcumin in proceeding through mutated genes in those pathways. In this study, the efficacy of curcumin was investigated in DBTRG cells. The cytotoxic ability was detected with MTT assay, and the influence of the cell cycle was checked with flow cytometry. The influence of the core signaling pathways was evaluated by Western blotting through the predominantly mutated proteins which included p53, p21, and cdc2 in the p53 pathway, CDKN2A/p16 and RB in the RB pathway, and EGFR, mTOR, Ras, PTEN, and Akt in the RTK-Ras-PI3K pathway. In addition, the apoptotic effect was determined by apoptosis-associated proteins Bcl-2, Bax, and caspase 3. Curcumin exhibits superior cytotoxicity on glioblastoma in a dose- and time-dependent manner in the MTT assay. In the core signaling pathways of glioblastoma, curcumin either significantly influences the p53 pathway by enhancing p53 and p21 and suppressing cdc2 or significantly inhibits the RB pathway by enhancing CDKN2A/p16 and suppressing phosphorylated RB. In the apoptotic pathway, the Bax and caspase 3 are significantly suppressed by curcumin and the Giemsa stain elucidates apoptotic features of DBTRG cells as well. In conclusion, curcumin appears to be an effective anti-glioblastoma drug through inhibition of the two core signaling pathways and promotion of the apoptotic pathway.

Introduction

Curcumin is a natural polyphenol compound and derived from several curcuma species, such as turmeric. It has been widely used in the human diet for centuries (1). However, its medical applications have been broadly investigated and explored, including its anti-cancer, anti-inflammation, and anti-angiogenesis activities. The anti-cancer effects of curcumin involve apoptosis of cancerous cells, cell cycle arrest, suppression of oncogenes, and promotion of tumor suppressor genes (2,3). Furthermore, several anti-cancer mechanisms and pathways of curcumin on human cancers have been identified. As in the most prevalent cancers, curcumin can suppress EGFR of colon cancer cells in a dose-dependent manner and inhibit the activity of EPGR promoters through reducing DNA-binding activity of egr-1 (4). In prostate cancer, curcumin induces apoptosis through hypersensitivity to tumor necrosis factor-related apoptosis-inducing ligand, which is mediated by inhibition of phospho-Akt and subsequently reductive transcription of nuclear factor (NF)-κB (5). In breast cancer, curcumin inhibits cancer cell proliferation and migration by increasing the Bax to Bcl-2 ratio and decreasing NF-κBp65 in a dose-dependent manner (6).

In malignant brain tumors, curcumin also exhibits its pleiotropic activity, which includes inducing cell cycle arrest at the G2/M stage through up-regulation of ING4, suppressing tumor growth by inhibition of AP-1 and NF-κB, and inhibiting anti-apoptotic signaling by increasing the Bax to Bcl-2 ratio (7-9). Nevertheless, most failure of cancer therapy in clinical practice is attributed to the polymorphism of cancer cells. To overcome this dilemma, a comprehensive exploration of genetic aberrations of cancer is not just helpful, but is required. The Cancer Genome Atlas have reported their research regarding the aberrations of DNA copy number, gene expression, and DNA methylation of glioblastoma multiforme (GBM) and concluded that there were three core aberrant signaling pathways, including RTK/RAS/PI3K, p53, and RB signaling pathways (10). Several genes were identified to be
more frequently mutated in those pathways, such as EGFR and PTEN in the RTK pathway, CDKN2A(ARF) and TP53 in the p53 pathway, and CDKN2A(p16/INK4A) and CDKN2B in the RB pathway. In addition, adult malignant gliomas have been reviewed, and genetic alterations of different pathological types have been summarized. Among the anaplastic astrocytoma and GBM, the predominant mutated genes include EGFR, PTEN, MDM2, TP53, RB, and CDKN2A(INK4A), which are comparable to the discoveries of The Cancer Genome Atlas (11). To explore the ultimately effective anti-glioblastoma activity of curcumin, we investigated the mechanism emphasized in the three mentioned core pathways.

Materials and methods

Preparation of reagents. Curcumin was purchased from Herbasion (Shenyang, China), and aprotinin, antipain, sodium deoxycholy, leupeptin, propidium iodide (PI), sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A, and MTT [3-(4,5)-dimethylthiahiazol-(z-y1)-3,5-di-phenyl-tetrazoliumromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates, and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Preparation of human GBM cell line DBTNTR. The human GBM cell line DBTNTR was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were placed into 75-cm² tissue culture flasks and incubated with RPMI-1640 medium (Gibco BRL) containing 10% heat-inactivated fetal bovine serum, (FBS) (Gibco BRL), 1% penicillin-streptomycin, 1% HEPES (Gibco BRL), and 1% sodium pyruvate (Gibco BRL). Cells were grown at 37°C in humidified atmosphere (5% CO₂). All data presented in this report are from at least three independent experiments.

Effects of curcumin on the viability of DBTNTR cells. The DBTNTR cells were plated in a density of 1x10⁴ cells/well and grown for 24 h. Increased concentrations of curcumin 0, 10, 20, to 30 μg/ml were added and cells were grown for 24 and 48 h at 37°C, while the 0 μg/ml-treated cells were set as control group and 0.2% DMSO (solvent) was added to these. For determining cell viability, after 24 and 48 h culture, the viability was evaluated by MTT assay and repeated four times. Briefly, DBTNTR cells were seeded in a 96-well plate in a density of 1x10⁴ cells/well and allowed to adhere overnight. After removing the medium, 200 μl of fresh medium containing 10 mM HEPES was added to each well, along with 20 μl 3-(4,5)-dimethylthiazol-2-y1)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution also added to each well. Then, the plate was incubated for 2 h at 37°C in the dark. After removing the medium, 100 μl DMSO was added into each well. The absorbance was measured using an ELISA plate reader at 590 nm.

Cell cycle analysis for DBTNTR cells treated with curcumin by flow cytometry assay. The percentage of cells in sub-G1, G0/G1, S, and G2/M phases were determined by flow cytometry as described in a previous report (12). Briefly, 2x10⁶ DBTNTR cells/10-cm dish were incubated with increased concentrations of curcumin, 0, 8, 16, and 32 μg/ml for 24 h before cells were harvested by centrifugation. In addition, 2x10⁶ DBTNTR cells/10-cm dish were incubated with 16 μg/ml curcumin for 0, 12, 24, and 48 h. Cells, after being harvested, were washed with PBS then fixed gently (drop by drop) in 70% ethanol. Cells were put in an ice bath overnight then re-suspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase (Sigma), and 0.1% Triton x-100. After 30 min incubation at 37°C in the dark, cells were analyzed with flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. Cell cycle and apoptosis were determined and analyzed using ModFit software. The average of the percentage of each phase in the cell cycle is representative of three independent experiments.

Preparation of proteins. About 2x10⁶ DBTNTR cells in a 10-cm dish were incubated with increased concentrations of curcumin, 0, 8, 16, to 32 μg/ml for 24 h before cells were harvested by centrifugation. In addition, ~2x10⁶ cells in a 10-cm dish were incubated with curcumin at 16 μg/ml concentration for increased durations of 0, 12, 24, and 48 h before cells were harvested by centrifugation. Protein was extracted as described previously (13). Briefly, cell pellets were re-suspended in Pro-Prep buffer (Intron Biotechnology, Inc.) for 30 min at 4°C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4°C and the supernatant was collected and stored at -80°C until assay. Protein concentrations were measured with the Bradford method (14).

Western blotting for examining the effect of curcumin on the expression of cancer-related proteins. The samples were separated by sodium dodecylsulfate polyacrylamide (SDS-PAGE) gel electrophoresis (10, 12.5, and 15%) (Bio-Rad Life Science Products, Hercules, CA, USA) as described (6). The SDS-separated proteins were followed by equilibration in transfer buffer [50 mM Tris, pH 9.0, 40 mM glycine, 0.175% SDS, 20% methanol and electro transferred to Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA, USA)]. Then, the blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl Sigma Chemical Co.) containing 0.1% Tween-20 for 30 min. After that, the blot was washed and incubated with antibodies to caspase 3 (R&D), Bax (Upstate), Bcl-2 (R&D), p53 (Sigma), p21 (Upstate), m-TOR, Ras, PTEN, Akt, phosphated Akt, CDKN2A/p16 (Abcam), RB, and ß-actin (Upstate) at 4°C overnight. After incubating with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the signal was visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Detection of ß-actin was used as an internal control in the data of Western blotting.

Giemsa stain. DBTNTR cells were plated at a density of 1x10⁴ cells/well and grown for 24 h. Increased concentrations of curcumin, 0, 8, 16, and 32 μg/ml were added and cells were cultured under two conditions, 24 or 48 h. After removing the culture medium, dishes were washed twice with PBS and fixed with Carnoy’s fixation solution (methanol and acetic acid)
acid mixture, 3:1) for 10 min at room temperature. After, the fixation solution was removed. Dishes were washed twice with PBS again and added in 10% Giemsa solution for staining. After 2-3 min incubation at room temperature, dishes were washed with PBS then examined under light microscope and photographed. In addition, the time-dependent model was performed with increased durations of 0, 24, and 48 h.

Statistical analysis. Data were depicted with mean value ± standard deviation. The untreated group (0 μg/ml) was set as the control group. The value (detected-protein/β-actin) of the control group in Western blotting was adjusted to 100% and others (various concentrations and durations) were adjusted to the same ratio. The difference between the respective groups was compared with one-way ANOVA and Bonferroni post test, and p-values <0.05 were considered a significant difference for all tests.

Results

Cytotoxicity of curcumin on DBTRG cells by MTT assay. The MTT assay was performed in dose- and time-dependent modes. When the curcumin dose increased, the survival of DBTRG cells decreased gradually either after 24 or 48 h treatment by curcumin (Fig. 1A and B). The IC50 was ~16.1 μg/ml after 24 h treatment and 11.2 μg/ml after 48 h treatment. In the time-dependent mode, dramatic cell death developed after 48 h treatment of 20 μg/ml curcumin (Fig. 1C). These results indicate that curcumin exerts cytotoxic activity on DBTRG cells in a manner that is both time- and dose-dependent.

Morphological change of DBTRG cells treated with increased concentrations of curcumin. The morphological change of DBTRG cells was inspected after treatment with curcumin with increased concentrations and durations. Cells shrank from a flat and elongated shape to a small and unevenly round shape after treatment with curcumin. Adhesively viable cells decreased as the treatment dose increased (Fig. 2A-D) or treatment duration increased (Fig. 2E-H).

Cell cycle arrest induced by curcumin. The cell cycle of DBTRG cells was analyzed by flow cytometry after the treatment of curcumin with increased concentrations and durations. The cytometric histograms exhibited arrest of the cell cycle at the G2/M phase after treatment, compared with the
Figure 3. Flow cytometry of DBTRG cell cycle affected by the treatment of curcumin. A-D, DBTRG cells treated with various concentrations of curcumin, 0 (non-treated), 8, 16 and 32 μg/ml for 24 h. The first pick waveform represents cells in G0/G1 phase and the secondary pick waveform represents cells in G2/M phase. E-H, DBTRG cells treated by various dosages of curcumin, 0 (non-treated), 8, 16, and 32 μg/ml for 48 h. I-L, DBTRG treated with 16 μg/ml curcumin with increased durations, 0 (non-treated), 12, 24, and 48 h.

Figure 4. The effect of curcumin on the RB pathway. A, Left, Western blotting and histogram of CDKN2A/p16 with various dosages of curcumin; and right, phosphorylated RB. B, Western blotting and histogram of CDKN2A/p16 and phosphorylated RB treated with curcumin in various durations. *p<0.05 compared with control.
non-treated, either in 24 or 48 h (Fig. 3A-H). The population of G2/M phase cells increased 2-3-fold after treatment in the 24-h group and 3-4-fold in the 48-h group. When DBTRG cells were treated with IC50 (16 μg/ml), the G2/M phase cells increased nearly 2-fold (I-L).

Effects of curcumin on aberrant-expression proteins in core signaling pathways of GBM. Three core signaling pathways were investigated, including the p53 pathway, RTK-RAS-PI3K pathway, and RB pathway. In total, 8 cancer-related proteins were evaluated for the effect of curcumin on their expression in DBTRG cells, which were p53 and p21 in the p53 pathway (Fig. 5), CDKN2A/p16 and RB in the RB pathway, and Ras, mTOR, PTEN, and ATK in the RTK-RAS-PI3K pathway (Fig. 6). In the RB pathway, CDKN2A/p16 exhibited up-regulated and phosphorylated RB exhibited increased ratio to total RB after curcumin treatment either by increased treatment dosage or duration, which would result in arrest of the cancerous cell cycle at the G1/S stage and senescence or apoptosis of the cancerous cells subsequently (Fig. 3A and B). In the p53 pathway, p53 and p21 were up-regulated by administration of curcumin either by increased treatment dosage or duration, which induced intrinsic apoptosis or arrest of the cancerous cell cycle at the G2/M stage (Fig. 4A and B). In the pathway of RTK-RAS-PI3K, PTEN showed no obvious influence by curcumin except in higher concentrations (32 μg/ml). Additionally, there was no significant difference of the ratio of phosphorylated Akt to total Akt. Ras and mTOR were not detected by Western blotting. From the above results, two of three core pathways of GBM, p53 and RB, are significantly inhibited by curcumin (Figs. 5 and 6).

Effects of curcumin on apoptosis-related proteins. Three apoptosis-related proteins were evaluated for the effect of curcumin on their expression from DBTRG cells, which included Bcl-2, Bax, and caspase 3. Curcumin slightly
suppressed the expression of Bcl-2 but enhanced Bax expression apparently. To the downstream of the apoptosis pathway, caspase 3 was increased significantly, up to 2.5-fold when the therapeutic duration was sustained for >48 h (Fig. 7).

Apoptotic feature of DBTRG cells after curcumin treatment. Apoptosis of cells developed either after 24 or 48 h (Fig. 8A and B) treatment in increased-dosage mode, however, the morphological change became apparent when dosage went beyond the IC_{50} dose, 16 μg/ml. With the increase of therapeutic time, the apoptotic feature was displayed more abundantly (Fig. 8C).

Discussion

The anti-cancer mechanisms of curcumin in a malignant brain tumor involve cell proliferation, growth, survival, and apoptosis. Nevertheless, most previously identified mechanisms are related to the apoptotic pathway (9). For example, Pratibha and colleagues examined and compared the efficacy of three curcuminoids through Bcl-2-mediated apoptosis. Surajit and colleagues demonstrated the activity of curcumin by inducing receptor- and mitochondria-mediated apoptotic pathways (15,16). Besides the apoptotic pathway, Liu and colleagues claimed that curcumin exerts cancer resistance not through apoptosis but through arrest of the cell cycle at the G2/M phase and is mediated by up-regulation of p53 (8). Nevertheless, in this study, we explored the anti-cancer mechanism not only for apoptosis, but also documented the most prevalent genetic aberrance of GBM. It is apparent that curcumin exhibits a pleotropic anti-GBM phenomenon since curcumin could increase the Bax/Bcl-2 ratio to induce apoptosis and inhibit at least two cancer signaling pathways to induce arrest of the cell cycle at the same time.

In the Akt-related pathway, curcumin has been verified to inhibit phosphorylation of Akt in several cancer cell lines such as prostate cancer, leukemia, and even malignant brain tumor (5,9,17). Nevertheless, the phosphorylation of Akt was not influenced by curcumin significantly in this experimental cell line. For further realization of this conflict, we checked the upstream of Akt in this cell line including EGFR, Ras, mTOR, and PTEN. It was concluded that the Ras and mTOR exhibited undetectable expression in Western blotting, the EGFR displayed no obvious change, and PTEN showed increased levels only in a highly therapeutic dosage of curcumin. This contradictory result is due to the polymorphic genetic aberrations in cancer cells and may play an important role in the resistance of cancer therapy. To survey the efficacy of a treating reagent through a comprehensive cancer genetic abnormality is required.

Arrest of the cell cycle at the G2/M phase is one of the effects of curcumin found in this study, which is in agreement...
with our previous finding in breast cancer MDA-MB-231 cells, findings in human glioma U87 cells by Pratibha et al., and Liu et al in human glioma U251 cells (4,7,16). This event was considered to be derived from up-regulation of p53, which has been identified as a common target of curcumin in different cancers. For insurance of p53 functioning in the check-point of G2/M, we further approve up-regulation of p21 and down-regulation of cdc2 following the increasing of p53.

Within the core signaling pathway of GBM, the RB pathway could be inhibited by curcumin via up-regulation of CDKN2A/p16 and down-regulation of phosphorylated RB in this study. RB is a check-point of G1/S phase and is recognized as a tumor suppressor gene. Phosphorylation of RB induces dissociation of the RB-repressor complex and permits transcription of G1 gene for proceeding G1 to S phase (18). Inhibition of RB pathway and arrest of the cell cycle at G1/S
by curcumin has not been reported up to now except in one report, in which Shishir and colleagues demonstrated that curcumin suppressed NF-κB and IκBα to arrest the cell cycle at the G1/S phase by down-regulation of NF-κB-regulated genes, and induced apoptosis by activation of caspases 9, 7, and 3 (19). In summary, curcumin promotes both checkpoints of the cell cycle, at least in this cell line model.

In this anti-GBM study, the efficacy of curcumin was investigated, including cytotoxicity of GBM cells, change of cell cycle of GBM, genetic expression of GBM signaling pathway, and the apoptotic pathway. The MTT assay revealed a dose- and time-dependent cytotoxic phenomenon of curcumin on GBM. Definite inhibition on the p53 and RB pathways by curcumin were validated via enhancing the expression of tumor suppressor genes and suppression of oncogenes. Curcumin shows potential benefits for treatment of GBM.

References