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Inhibitory effects of curcumin on gastric cancer cells: A proteomic study of molecular targets

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ABSTRACT

Curcumin, a natural anticancer agent, has been shown to inhibit cell growth in a number of tumor cell lines and animal models. We examined the inhibition of curcumin on cell viability and its induction of apoptosis using different gastric cancer cell lines (BGC-823, MKN-45 and SCG-7901). 3-(4,5-dimethyl-thiazol-2-yl)-2-5-diphenyltetrazolium-bromide (MTT) assay showed that curcumin inhibited cell growth in a dose- (1, 5, 10 and 30 μM) and time- (24, 48, 72 and 96 h) dependent manner; analysis of Annexin V binding showed that curcumin induced apoptosis at the dose of 10 and 30 μM when the cells were treated for 24 and 48 h. As cancers are caused by dysregulation of various proteins, we investigated target proteins associated with curcumin by two-dimensional gel electrophoresis (2-DE) and MALDI-TOF-TOF mass spectrometer. BGC-823 cells were treated with 30 μM curcumin for 24 h and total protein was extracted for the 2-DE. In the first dimension of the 2-DE, protein samples (800 μg) were applied to immobilized pH gradient (IPG) strips (24 cm, pH 3–10, NL) and the isoelectric focusing (IEF) was performed using a step-wise voltage ramp; the second dimension was performed using 12.5% SDS-PAGE gel at 1 W constant power per gel. In total, 75 proteins showed significant changes over 1.5-fold in curcumin-treated cells compared to control cells (Student's *t*-test, $p < 0.05$). Among them, 33 proteins were upregulated and 42 proteins downregulated by curcumin as determined by spot densitometry. 52 proteins with significant mascot scores were identified and implicated in cancer development and progression. Their biological function included cell proliferation, cycle and apoptosis (20%), metabolism (16%), nucleic acid processing (15%), cytoskeleton organization and movement (11%), signal transduction (11%), protein folding, proteolysis and translation (20%), and immune response (2%). Furthermore, protein–protein interacting analysis demonstrated the interaction networks affected by curcumin in gastric cancer cells. These data provide some clues for explaining the anticancer mechanisms of curcumin and explore more potent molecular targets of the drug expected to be helpful for the development of new drugs.

Introduction

Gastric cancer is one of the most common types of cancer and the malignancy is the second leading cause of cancer mortality worldwide (Wagner and Wedding, 2009). Growing evidence shows that the pathogenesis of gastric cancer is complex and related to multiple oncogenic pathways, such as those mediated by cell cycle regulators, nuclear factor-kappaB (NF-κB), epidermal growth factor receptor (EGFR) and STATs are also involved in gastric cancer (Ernst et al., 2008; Huang et al., 2011; Wu et al., 2010). Many small molecule inhibitors targeting these signal pathways are vigorously developed, and some have been demonstrated to be effective in clinical settings (Asaoka et al., 2011; Magan et al., 2012; Siegel-Lakhai et al., 2005).

As natural anticancer agents, curcumin and its analogs have been paid more attention because of their potent inhibitory effect on tumor and their medicinal properties in Indian and Chinese systems of medicine. More recently curcumin has been found to inhibit multiple cancers via its effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis (Wilken et al., 2011). The biological functions influenced by this agent are owed to its various molecular targets, including transcription factors, growth factors and their receptors, cytokines, enzymes, and genes regulating cell proliferation and apotosis (Kunnumakkara et al., 2008). For instance, curcumin induced EGFR degradation in lung adenocarcinoma and modulated p38 activation in intestine (Lee et al., 2011); C086, a novel analog of curcumin, induced growth inhibition and down-regulation of NF-κB in colon cancer cells and xenograft tumors (Chen et al., 2011); its other analogs also exhibited potent suppressive activity on breast and prostate cancer via signal STAT3 inhibition (Lin et al., 2009; Wang et al., 2011) Although some

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anticancer mechanisms of curcumin have been studied, much remains to be delineated for its effect on gastric cancer.

With advances in technology, two-dimensional polyacrylamide gel electrophoresis (2-DE) is used to examine heterogeneity of protein expression in cells under different conditions. 2-DE combined with MALDI-TOF-MS has been applied in comparative proteomics to identify anticancer mechanisms. Here we investigated the anticancer effect of curcumin, and employed comparative proteomic strategies to identify differentially expressed proteins associated with curcumin and to offer new insights into the anticancer mechanisms.

Methods

Cell culture and reagents

Curcumin (diferuloylmethane) was purchased from Sigma–Aldrich Corporation and was prepared with dimethyl sulfoxide (DMSO) at the concentration of 20 mM, stored as small aliquots at −20 °C, and thawed and diluted as needed in cell culture medium. Four different human gastric cancer cell lines (BGC-823, MKN-45, SCG-7901) were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (Invitrogen) at 37 °C in incubator with humidified atmosphere of 5% CO_2 and 95% air.

Cell proliferation assay

The cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium-bromide (MTT) (Sigma–Aldrich) assay. Cells were seeded in 96-well Corning plates at a density of 1×10^4 cells/well overnight, and then treated with the indicated doses of curcumin or DMSO for 24, 48, 72, 96 h and then 20 μl of MTT solution (5 mg/ml in PBS) was added to each well. After 4 h incubation at 37 °C, the medium was removed from the wells and 150 μl of DMSO was added. The absorbance was measured at 595 nm using a Microplate Reader (BIO-RAD). Growth inhibitory rate = [1-(A595 treated/A595 control)] × 100%. The experiments were carried out in triplicate and repeated three times.

Annexin V binding assays

Analysis of Annexin V binding was carried out using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™) according to the manufacturer's instructions. Briefly, cells were incubated with curcumin for 24 or 48 h (DMSO as control), and then harvested. Cells were washed twice with cold phosphate-buffered saline, and centrifuged at 1500 rpm for 5 min. Cells were resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml, and 100 μl of the solution was transferred into a 5 ml culture tube, and 5 μl of FITC Annexin V and 5 μl of propidium iodide (PI) were added. After a 15 min incubation at room temperature in the dark, 400 μl of 1× binding buffer was added to each tube, and samples were analyzed using FACScan flow cytometer (Becton Dickinson). For each sample, 10,000 events were acquired. The experiments were carried out in triplicate and repeated three times.

Sample preparation and 2-DE

Cells were cultured at a density of 1×10^6 cells per 10 cm plate overnight and treated with DMSO or curcumin for 24 h. Cells were then pelleted, washed with PBS and solubilized in 500 μl of a buffer composed of 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.5% IPG buffer and 1% protease inhibitor cocktail. A 2-D Clean-Up Kit was used to purify proteins from supernatant and then total protein concentration was measured by 2-D Quant Kit (GE Healthcare). In the first dimension of the 2-DE, protein samples (800 μg) were applied to immobilized pH gradient (IPG) strips (24 cm, pH 3–10, NL). Isoelectric focusing (IEF) was performed using a step-wise voltage ramp by IPGphor III system (GE Healthcare): 30 V for 12 h, 300 V for 3 h, linear ramping from 300 V to 1000 V for 6 h and from 1000 V to 8000 V for 3 h, and finally 8000 V for 7 h. Once IEF was completed, the strips were equilibrated in equilibration buffer (75 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 1% DTT) for 15 min, followed by the same buffer containing 2.5% iodoacetamide instead of DTT for another 15 min. The second dimension was performed using 12.5% SDS-PAGE gel (260 mm × 200 mm × 1 mm) at 1 W constant power per gel by Ettan DALTsix (GE Healthcare). The experiments were carried out in triplicate.

Protein visualization and image analysis

Proteins were visualized by 0.1% Coomassie brilliant blue R-350 (PhastGel™ Blue R). All gels were scanned on a UMAX PowerLook 2100XL scanner, and protein spots were analyzed quantitatively using the ImageMaster 2D Platinum 6.0 software (GE Healthcare). Triplicate runs were made to ensure the accuracy of analyses. Spots on the gels were matched and the volume percentage of each spot representing a certain protein was quantified and analyzed according to the Imagemaster software instructions. The quantity of each spot was normalized by the total valid spot intensity. Due to the intrinsic variability, we chose a stringent criterion: (i) a change of expression of at least 1.5-fold, (ii) *t*-test value ($p < 0.05$), and (iii) the identification of the spots in the three experimental replicates. The proteins displaying spot densitometry difference that were <1.5 changes may also be of interest, but arbitrary cutoff at >1.5 allowed us to focus on the most dramatic differences in protein spot expression.

In-gel digestion

Protein spots were automatically excised from the 2-D gels by Ettan Spot Picker (Amersham Biosciences). They were washed in distilled water twice, and then equilibrated with 25 mM ammonium bicarbonate in 50% ACN at 37 °C for 20 min. Finally, they were dehydrated in 100% ACN until gel plugs became opaque. Thereafter, vacuum-dried gel plugs were rehydrated with 10 μg/ml of trypsin (Promega) in 25 mM ammonium bicarbonate (pH 7.8). Proteolysis of proteins was performed at 37 °C for 16–18 h. Supernatants was collected and mixed with the HCCA (Bruker Daltonics) with 0.4 mg/ml in 70% ACN and 0.1% TFA. They were then spotted onto the target plate and allowed to dry.

MALDI-TOF/TOF mass spectrometer and database searches

An AutoFlex III (Bruker Daltonics) MALDI-TOF/TOF mass spectrometer was used with a mass accuracy of 50 ppm after external calibration. The samples were analyzed in the MS mode (for generation of peptide mass fingerprints) as well as in the TOF/TOF mode (for fragmentation analysis of the two or three most intense peaks). MS spectra were transformed into peak lists by using the software flexAnalysis version 3.0 (Bruker Daltonics). The peak lists of the MS and MS/MS spectra were merged by the software BioTools version 3.0 (Bruker Daltonics). Proteins were identified using the protein identification software program MASCOT (Matrix Science) against Swiss-Prot database (Swiss Institute of Bioinformatics). During the data search, maximum allowance was set with one missed cleavage per peptide; MS/MS tolerance of 0.4 Da and a mass tolerance of 50 ppm were also used according to predefined optimized protocol. Carbamidomethylation for cysteine, oxidation for methionine and other variants were also taken into consideration. Before the search of fragment in the database, manual quest