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Differential anti-tumor activities of curcumin against Ras- and Src-activated human adenocarcinoma cells

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ABSTRACT

Although curcumin has been studied as a potential anticancer drug targeting multiple signaling molecules, the role of oncogenic Src and Ras in curcumin sensitivity remains unknown. Using HAG-1 human adenocarcinoma cells transfected with either activated Src or Ras, we investigated here the functional role of these oncogenes in curcumin sensitivity. Activation of either Src or Ras did not confer resistance to curcumin, compared to vehicle-transfected cells. Curcumin enhanced Erk1/2 predominantly in Ras-activated cells, but inhibited Akt and its downstream molecules (mTOR and S6K1) regardless of these oncogene activations. The sub-G0/G1 apoptotic populations were substantially increased with demonstrable cleavage of PARP, but this increase was most prominent in Src-activated cells. Suppression of Bcl-xL level and enhanced expression of Bax were demonstrated in Src-activated, but not Ras-activated cells. By contrast, drastic increases of G_2/M cell populations were seen in Ras-activated cells rather than Src-activated cells, suggesting a potential role of Ras/Erk1/2 activation in curcumin-induced G_2/M arrest. These data indicate that curcumin-induced growth inhibition would be mediated mainly by G_2/M arrest in Ras-driven cells but by apoptosis induction in Src-driven cells, providing a mechanistic rationale for the potential use of curcumin in the treatment of human cancers with activated Src or Ras.

1. Introduction

Ras and Src are crucial oncogene products implicated in the pathogenesis of many human cancers. Activation of Ras through point mutation was most frequently identified in a variety of human cancers, including adenocarcinoma of the pancreas, colon, and lung [1]. Ras transmits a signal to the serine/threonine kinase Raf, which subsequently activates mitogen-activated protein (MAP) kinase, resulting in cell proliferation through the transcriptional activation of a variety of targets [2]. Activation of Src as detected by the elevation of Src tyrosine kinase activity was also identified in a variety of human cancers, such as breast, colon, skin, bladder, and pancreas cancer [3]. Specifically, Src has been found to be highly activated in colon cancer metastasized to the liver [4]. Src phosphorylates a number of intracellular substrates on tyrosine residue [5], resulting in a generation of mitogenic and anti-apoptotic signals from Src to downstream signalings including not only Ras-Raf-Erk1/2, but also PI3K-Akt-mTOR pathways. Currently, no molecular targeted therapy exists that would be effective against human solid tumors having either activated Ras or Src.

Curcumin is derived from turmeric (*Curcuma longa*) and is a natural polyphenol. Curcumin has long been used as a food, coloring agent, and traditional medicine. It is safe and nontoxic, and has demonstrable anti-inflammatory and antioxidant properties [6]. Curcumin has been shown to inhibit the formation of carcinogen-induced cancers in rodents [7–11]. Moreover, increasing evidence indicated that curcumin has anticancer effects against a variety types of human tumor cells through modulation of diverse molecular targets involving cell survival/apoptosis and proliferation [6]. Specifically, the epidermal growth factor receptor (EGFR) tyrosine kinase has been reported as a potential target of curcumin [12,13]. Curcumin inhibits the EGFR intrinsic kinase activity in human epidermoid [13], breast [14], prostate [15,16], and colon cancer [17]. Curcumin has been shown to block EGFR signaling by preventing EGFR tyrosine phosphorylation and suppressing EGFR gene expression [18].

Ras and Src are major signaling molecules that share downstream signaling pathways with EGFR. Therefore, it is interesting to know whether activation of downstream signalings of EGFR may influence the sensitivity to curcumin. However, there has been no report that investigates the functional role of Ras and Src in curcumin-induced growth-inhibition and apoptosis in human cancer cells. Since the role of such oncogenic signalings in the curcumin sensitivity remains to be clarified, we have

Abbreviations: 4E-BP1, eukaryotic initiation factor 4-binding protein 1; Cdk1, cyclin-dependent kinase 1; EGFR, epidermal growth factor receptor; FACS, fluorescence activated cell sorting; IC_{50}, 50% inhibitory concentration; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PARP, Poly ADP-ribose polymerase; PI, Propidium iodide; PI3K, phosphoinositide 3-kinase; S6K1, p70-S6 kinase 1.

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investigated here the mechanistic role of Src and Ras, in curcumin sensitivity, specifically through Akt and Erk1/2 pathways as well as apoptosis-associated proteins using EGFR-expressing HAG-1 human gallbladder carcinoma cell lines transfected with activated Ras and Src.

2. Materials and methods

2.1. Cell culture and chemicals

HAG-1 is a human epithelial cell line derived from a moderately differentiated adenocarcinoma of the gallbladder [19]. No mutations and amplifications of H-, K-, or N-*ras* genes have been detected. This cell line has been demonstrated to substantially express EGFR by flow cytometric analysis [20]. The HAG/ras5-1 cells were obtained by transfecting HAG-1 parental cells with activated c-H-*ras*, while HAG/src3-1 cells that express $p60^{v\text{-}src}$ protein were obtained by transfection of the pSV2/v-*src* into HAG-1 cells [21]. HAG/neo3–5 cells were obtained by transfection of HAG-1 cells with pSV2neo alone, which carries the gene for neomycin resistance, and used as a vehicle control. v-Src has a constitutively activated tyrosine kinase activity by the lack of negative regulatory domain. These cells were cultured in Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Carlsbad, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. HAG/ras5-1 and HAG/src3-1 cells were grown in the same conditions, except that G418 (200 μg/ml) was added to the culture medium.

Curcumin (more than 80% purity) was purchased from Sigma–Aldrich (St. Louis, MO, USA). A 100 mM solution was prepared by dissolving original curcumin powder with dimethyl sulfoxide (DMSO, Sigma–Aldrich) and subsequently with 100% ethanol as described [22]. The final concentration of DMSO for all experiments and treatments (including controls, where no drug was added) was maintained at less than 0.05%. These conditions were found to be non-cytotoxic for 72 h.

2.2. Determination of growth inhibition and apoptosis assessment by PARP cleavage

The anti-proliferative effects of curcumin on vehicle- and oncogene-transfected HAG-1 cells were assessed by WST assay [22]. Briefly, 100 μl suspension of HAG-1 cells was seeded into each well of a 96-well plate (BD Falcon, Franklin Lakes, NJ, USA) at a density of 2000 cells per well. After overnight incubation, 100 μl curcumin solutions at different concentrations were added and cells further cultured for 72 h. Cell viability was then measured by Premix CCK-8 Cell Proliferation Assay System (Dojindo, Japan). Each experiment was performed using six replicate wells for each curcumin concentration and was carried out independently three times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance.

Apoptosis was assessed by PARP cleavage detected by Western blot using antibody to PARP (9542). PARP is a substrate for certain caspases activated during early stages of apoptosis. These proteases cleave PARP to fragments of approximately 89 kDa and 24 kDa. Detection of the 89 kDa PARP fragment with antibody to PARP thus serves as an early marker of apoptosis.

2.3. Cell cycle analysis and apoptosis measurement

At various times following treatment with or without curcumin, floating and trypsinized adherent cells were combined, fixed in 100% ethanol and subjected to cell cycle analysis on a Beckman Coulter Gallios Flow Cytometer using the Kaluza ver. 1.2 software packages (Beckman Coulter, Brea, CA, USA). The extent of apoptosis was determined by measuring the sub-G_1 population.

2.4. Immunoprecipitation and Western blot analysis of signaling proteins

The cells were washed twice with ice-cold PBS and scraped into 0.5 ml of lysis buffer (10× Cell Lysis Buffer, 1 mM PMSF). After removal of cell debris by centrifugation, protein concentrations of the supernatants were determined by using a BCA protein assay kit (Bio Rad). Immune complexes were boiled in electrophoresis sample buffer (Bio Rad).

For Western blot, equal amounts of proteins or immunoprecipitated target proteins were resolved by 4–15% SDS–PAGE and electro transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ, USA). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min. The membranes were then incubated with primary antibodies against either phospho-mTOR (Ser2448), phospho-p44/42 Erk1/2 (Thr202/Tyr204, phospho-Akt (Ser473), phospho-S6K1 (Thr389), Bcl-xL (2762), or Bax (2772). The membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (7074). Immunoblots were developed with the enhanced chemiluminescence system (GE Healthcare) and were then quantitated using LAS-3000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan). The blots were striped and reprobed with primary antibodies against mTOR (9964), MAPK (9102), Akt (9272), and β-actin (4967). All primary and secondary antibodies were purchased from Cell Signaling Technology. For reblotting, membranes were incubated in stripping buffer (Thermo) for 30 min at room temperature before washing, blocking, and incubating with antibody. Triplicate determinations were made in separate experiments.

2.5. Statistical analysis

To determine the significance of observed differences, analysis of variance (ANOVA) was applied to the cell cycle data using statistical software (version 12.0.1 for Windows, SPSS Inc., U.S.A.). The mean values of cell cycle percentages were compared by Dunnett *t*-test. A p value less than 0.05 was considered significant.

3. Results

3.1. Effects of curcumin on proliferation and survival of HAG/neo3-5, HAG/src3-1, and HAG/ras5-1 cells

Curcumin treatment for 72 h exhibited dose-dependent antitumor activity against these cell lines (Fig. 1). The 50% inhibitory concentrations (IC_{50}) for 72 h exposure of curcumin were 22.4 ± 1.1 μM for HAG/neo35 cells, 23.2 ± 3.8 μM for HAG/ras5-1 cells, and 26.0 ± 0.2 μM for HAG/src3-1 cells, respectively, indicating no significant differences of curcumin sensitivity between these cell lines ($p = 0.120$).

3.2. Time-course analysis of the effect of curcumin on cell cycle progression and apoptosis

To examine whether the inhibitory effects observed in cytotoxicity assays reflect the arrest or delay of cell cycle or apoptotic cell death, cells were treated with curcumin at a dose of 25 μM, and the cell cycle progression and apoptosis were evaluated by FACS analysis. When HAG/neo3-5 cells were treated with curcumin, the proportion of cells in a G_2/M phase significantly increased from 20% at