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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

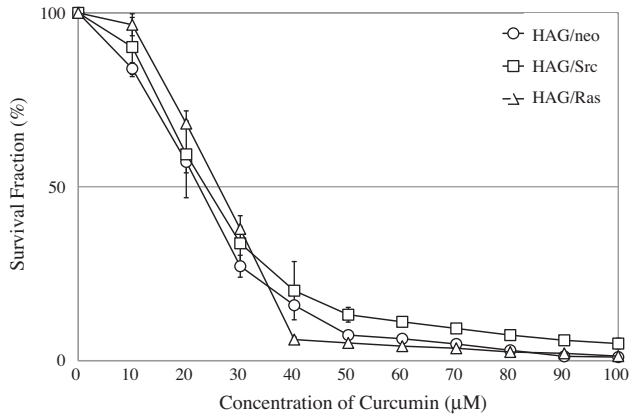

Fig. 1. Cytotoxicity of curcumin against HAG/neo (open circle), HAG/src3-1 (open square), and HAG/ras5-1 (open triangle) cells. Cells were treated with various concentration of curcumin for 72-h and assessed for cytotoxicity by WST-1 assay as described in Materials and methods. The data represent the means from three independent experiments. Bars, standard deviation.

the beginning of the treatment to 31%, with corresponding decrease in cells in G_0/G_1 phase, but the proportions of cells in each cell cycle phase did not significantly changed afterwards (Fig. 2A). The percentages of sub-G_0/G_1 cell population, which represents apoptotic cells, increased gradually after 24 h (3.4%) and 48 h (8.1%) exposures, and increased up to 18.9% 72 h post-treatment. When HAG/src3-1 cells was treated with curcumin, the proportion of cells in a G_2-M phase significantly decreased from 48 h, and did not change afterwards, with corresponding increases in the population of G_0/G_1 phase (Fig. 2B). The sub-G_0/G_1 apoptotic cell population substantially increased after 24 h exposure (10.3%) and increased nearly two-fold every 24 h, resulting in 40.5% after 72 h exposure (Fig. 2B). By contrast, when HAG/ras5-1 cells were treated with curcumin, the proportion of cells in a G2-M phase drastically increased from 20% to 52% after 24 h exposure, with corresponding decreases in cells in S and G_0–G_1 phase, and reached a plateau afterwards, while the sub-G_0/G_1 apoptotic cell population increased gradually after 24 h (2.5%), 48 h (6.4%) 72 h (14.8%) exposures (Fig. 2C). These data indicate that curcumin-induced growth decline would be mediated mainly by G2/M arrest of the cell cycle for Ras-activated cells. Percentages of curcumin-induced apoptosis were significantly higher in HAG/src3-1 cells than either HAG/neo3-5 ($p < 0.001$) or HAG/Ras5-1 cells ($p < 0.001$), indicating that progressive expansion of apoptotic cells would be the main reason for curcumin-induced cytotoxicity in Src-activated cells.

3.3. Effects of curcumin on activations of signaling molecules for cell proliferation and survival

Because activations of signaling molecules such as Erk1/2, Akt, and mTOR have been considered as major factors contributing toward proliferation and survival, we examined the effects of curcumin on expression or activation (phosphorylation) of these proteins in HAG/neo3-5, HAG/ras5-1, and HAG/src3-1 cells. Upon treatment with curcumin, the constitutive activity of Erk1/2 was temporarily enhanced only at 2 h treatment in vehicle-transfected HAG-1 cells and enhanced after 12 h treatment in HAG/src3-1 cells (Fig. 3A). The enhancement of Erk1/2 activity was most prominent in Ras-activated cells rather than control or Src-activated cells. By contrast, the phosphorylation of Akt and its downstream molecules (mTOR and S6K1) was significantly inhibited, irrespective of Src or Ras activation (Fig. 3A), indicating that curcumin can overcome Src- or Ras-driven activation of downstream signaling pathways.

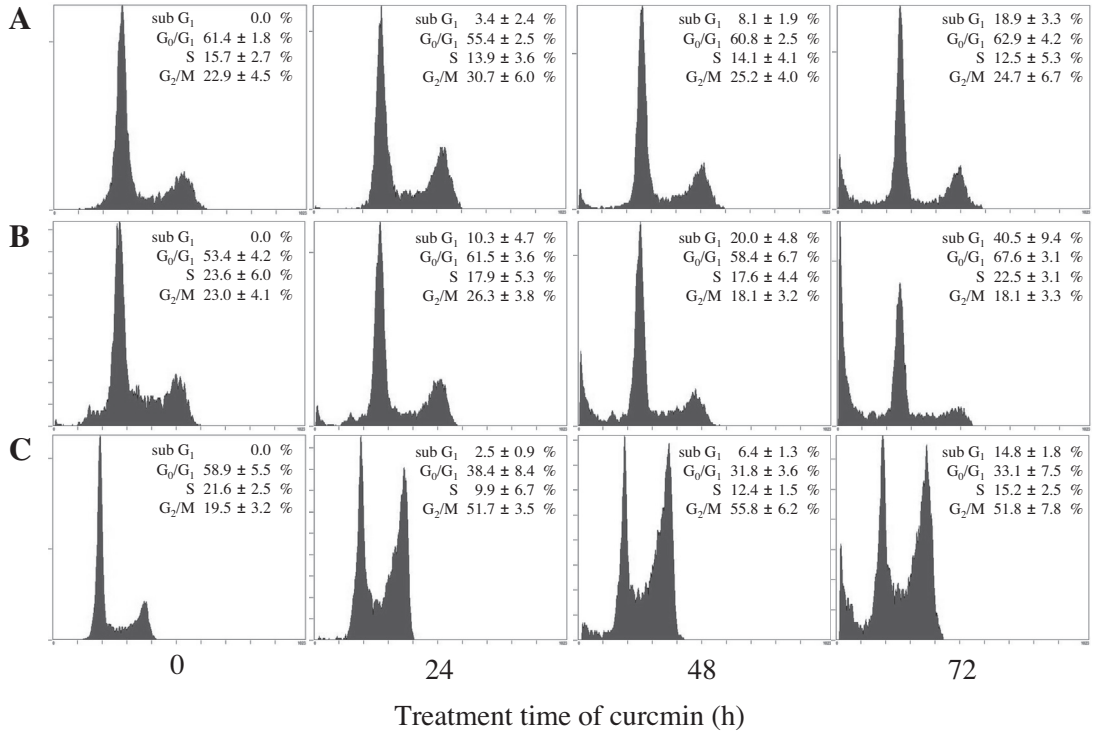

Fig. 2. Time-course analysis of the effect of curcumin on cell-cycle progression and apoptosis determined by flow cytometry. Representative cell-cycle distributions after exposure to 25 μM curcumin for 0, 24, 48, and 72 h were shown for HAG/neo (A), HAG/src3-1 (B), and HAG/ras5-1 (C). Percentages of the total cell population in the different phases of cell cycle were determined with curve fitting using the Kaluza ver. 1.2 software, and the mean values ± SD of three independent experiments are shown on the top right of each panel.

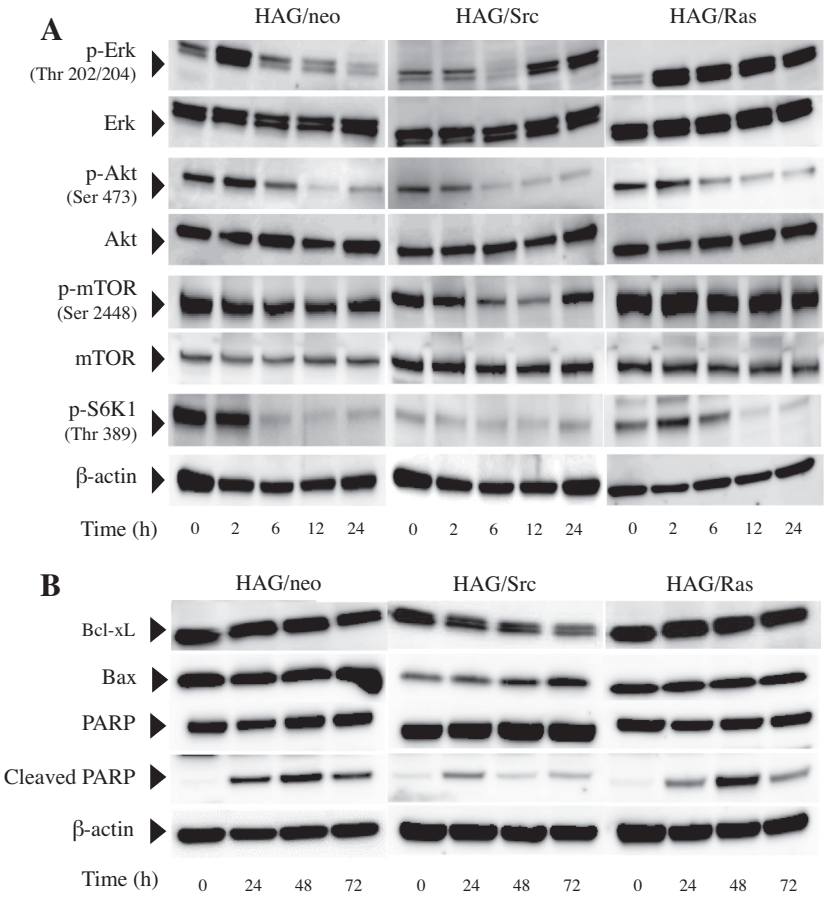

Fig. 3. Effects of curcumin on activations of signaling molecules for cell proliferation and survival in HAG/neo, HAG/src3-1, and HAG/ras5-1. (A) Western blots are shown for phosphorylated and total ERK1/2, AKT and mTOR. Phosphorylated S6K1 are also shown. (B) Effects of curcumin on the apoptotic and anti-apoptotic proteins including Bax and Bcl-xL. PARP and cleaved PARP are shown as an early marker of apoptosis.

3.4. Effects of curcumin on expression of Pro- and Anti-apoptotic proteins

Cleavage of PARP was demonstrated from 24 h to 72 h post-treatment in all three cell lines treated with curcumin (Fig. 3B). The band densities of Western blot for PARP cleavage in HAG/Src3-1 cells appear less than those in HAG/neo and HAG/ras5-1 cells. This is probably due to the anchorage-independent property of HAG/src3-1 cells that are easily detached from the substrate, remaining non-apoptotic attached cells in the plates.

To clarify the apoptotic mechanism induced by curcumin, we examined the expression of anti-apoptotic Bcl-xL as well as pro-apoptotic Bax (Fig. 3B). Upon treatment with 25 μM curcumin, expression of Bcl-xL was inhibited in a time-dependent manner in HAG/src3-1 cells, but not changed in HAG/neo3-5 cells and HAG/ras5-1 cells. Expression of pro-apoptotic Bax protein did not change with the incubation time with curcumin in HAG/neo3-5 cells and HAG/ras5-1 cells, but enhanced in HAG/Src3-1. Reduced expression of Bcl-xL and enhanced expression of Bax would be responsible for the curcumin-induced apoptotic induction in Src-driven cells. Bcl-xL and Bax appear not to be involved in curcumin-induced apoptosis in Ras-driven cells, providing explanations for less amounts of apoptosis induction in these cells rather than Src-driven cells.

4. Discussion

Curcumin has been shown to have anticancer effects against a variety types of human tumor cells through modulation of diverse molecular targets involved in cell survival/apoptosis and proliferation [6]. Specifically, curcumin has been shown to have the ability to inhibit EGFR tyrosine kinase activity [18]. Src and Ras are known to activate and share downstream signaling pathways of EGFR, and therefore may potentially abrogate the effect of curcumin. In order to prove this hypothesis, we investigated the functional role of activated Ras and Src in the curcumin sensitivity using EGFR-expressing HAG-1 human cells transfected either with activated c-H-Ras or v-Src. Unexpectedly, we have found that the IC_{50} of curcumin against HAG/neo5-3, HAG/ras5-1 and HAG/src3-1 were not significantly different, being 22, 26 and 23 μM, respectively. Since the IC_{50} of curcumin in parental HAG-1 cells was 25 μM [22], introduction of these oncogenes and vehicle transfection did not confer resistance to curcumin, making a striking difference from the results obtained with gefitinib, a selective inhibitor of EGFR tyrosine kinase, where the activation of Ras and Src induced marked resistance by nearly 30-fold and 200-fold, respectively, abolishing completely its apoptosis-inducing activity [23]. These data indicate that anticancer activity of curcumin appears to be mediated by the mechanism other than inhibition of EGFR tyrosine kinase activity.

We have found that curcumin-induced growth inhibitions are mediated by differential mechanisms of action, depending on the activation status of either Ras or Src. The curcumin-induced growth decline would be mediated by G_2/M arrest of the cell cycle in Ras-driven cells, while progressive expansion of apoptotic cell population would be the main reason for curcumin-induced growth decline in Src-driven cells. These distinct results indicate

that curcumin exhibits differential activities against apoptosis and cell cycle progression, depending on activation mechanisms of signal transduction. There are two major cell survival and growth signaling pathways downstream of Src and Ras, i.e., PI3K-Akt-mTOR and Ras-Raf-MAPK (Erk1/2) pathways. Activated Akt may positively regulate mTOR by directly phosphorylating mTOR at Ser2448, leading to increased phosphorylation of S6K1 [24], and play a critical role in controlling survival and apoptosis [25]. In many cancer cell lines, curcumin has been shown to inhibit phosphorylation of Akt/mTOR and its downstream targets, S6K1 and 4E-BP1, suggesting that curcumin may execute its anticancer effect primarily through blocking mTOR-mediated signaling pathways [26,27]. Accordingly, we have shown that curcumin inhibits the activities of of Akt, with subsequent reductions of activities of downstream mTOR and S6K1, regardless of activations of these oncogenes, indicating that curcumin can overcome the influences mediated by Src- and Ras-driven activation of downstream signaling pathways.

Cell cycle checkpoints are important control mechanisms that ensure the proper execution of cell cycle events. Curcumin has been shown to induce G_2/M arrest in many cancer cells [28–30]. The present study demonstrated that the blockade of cell cycle arrest at G_2/M was seen in Ras-activated cells, but not in control or Src-activated cells. Moreover, curcumin was shown to significantly enhance constitutive activities of Erk1/2. Interestingly, curcumin-induced enhancement of Erk1/2 activity was most prominent in Ras-activated cells rather than control or Src-activated cells. These data suggest that curcumin-induced G_2/M blockade of the cell cycle in Ras-activated cells might be due to Ras-driven augmentation of Erk1/2 activity. In this regard, treatment of colon, ovarian, and glioblastoma cell lines with curcumin has been shown to arrest the cells in the G_2/M phase and prevent cells cycle progression [28–30]. Since Ras is frequently activated in those cancers, observed curcumin-induced G_2/M blockade might be mediated by augmentation of Erk1/2 activity. The mechanism whereby activation of Erk1/2 induces G_2/M blockade remains unclear. However, activation of Erk1/2 has been demonstrated to correlate with changes in the level and/or activity of several key regulators of the G_2/M checkpoint, including Cdk1 (Cdc2) and Wee1 kinases [31,32]. Furthermore, inhibition of Erk1/2 signaling resulted in more than 85% attenuation of irradiation-induced G_2/M arrest [32]. Previous studies have also shown that concomitant decrease in the expressions and activity of Cdk1 (Cdc2) were seen in curcumin-treated cells [33]. Therefore, it is suggested that Ras activation enhances Erk1/2, and subsequently induces the change in these regulator molecules of G_2/M checkpoint, leading to the arrest of the cell cycle at G_2/M.

The mechanism whereby curcumin induces apoptosis still remains unclear. Curcumin was reported to induce the up-regulation of pro-apoptotic proteins from the Bcl-2 family (Bim, Bax, Bak) and the down-regulation of anti-apoptotic proteins (Bcl-2, Bcl-xL) [34,35]. In the present study, curcumin down-regulated expression of Bcl-xL in HAG/neo3-5 cells and HAG/Src3-1 cells, but unchanged in HAG/Ras5-1 cells. Expression of pro-apoptotic Bax was unchanged in HAG/neo3-5 cells and HAG/Ras5-1 cells, but enhanced in HAG/Src3-1 with incubation time, indicating that Bax contribution to curcumin-induced apoptosis appears to be involved in Src-driven cells, but not in vehicle control or Ras-activated cells. Therefore, it is suggested that usages of apoptotic proteins in curcumin-induced apoptosis may vary with cell type. Taken together, these data indicate that antitumor activity of curcumin would be mediated mainly by G2/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.

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