Curcumol Induces Senescence in Human Hepatocellular Carcinoma HepG2 Cells and Its Mechanism: Postprint

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Abstract

Abstract: Cellular senescence refers to the irreversible process by which cells transition from active proliferation into a state of growth arrest. Identifying drugs or molecular targets that induce tumor cell senescence may provide novel strategies for cancer therapy. Curcumol is a monomeric compound isolated from the traditional Chinese medicine Curcuma zedoaria that exhibits inhibitory effects on the proliferation of various human tumor cells. We previously reported that curcumol inhibits the proliferation of human hepatocellular carcinoma HepG2 cells. The present study further demonstrates that curcumol induces senescence-associated phenotypic changes in HepG2 cells, accompanied by G0/G1 phase cell cycle arrest. Using quantitative real-time PCR technology to analyze the differential expression profiles of 81 cell senescence-related genes in curcumol-treated cells, we found that the expression levels of TP53 and its downstream genes p16Ink4a, p21Waf1/Cip1, and p27Kip1 were significantly upregulated, accompanied by significantly enhanced transcription of multiple senescence signaling pathway initiation and effector-associated genes including ABL1, ALDH1A3, CHEK2, HRAS, and PTEN. Conversely, the expression levels of negative regulatory genes for cell cycle progression and senescence signaling pathways, such as Cyclin A2, IGFBP3, SIRT1, and TERT, were significantly downregulated. Western blot analysis revealed elevated levels of p53 and its downstream cyclin-dependent kinase inhibitor (CKI) molecules p21WAF1 and p16INK4, reduced Cyclin A2 levels, consistent with the PCR results, and accompanied by a significant increase in wild-type p53-induced protein phosphatase 1 (Wip1) levels, suggesting that curcumol may induce HepG2 cell senescence by activating the p53 signaling pathway. This study provides valuable information for further investigation of the anti-tumor mechanism of curcumol and its potential clinical applications.

Full Text

Preamble

Curcumol Induces Premature Senescence in Human Hepatocellular Carcinoma HepG2 Cells and Its Underlying Mechanisms

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Abstract

Cellular senescence is an irreversible process in which cells transition from active proliferation to growth arrest. Identifying drugs or molecular targets that can induce tumor cell senescence may provide novel therapeutic strategies for cancer treatment. Curcumol, a monomeric compound isolated from the traditional Chinese herb *Curcuma zedoaria*, exhibits inhibitory effects on the proliferation of various human tumor cells. We previously reported that curcumol suppresses the proliferation of human hepatocellular carcinoma HepG2 cells. The present study further demonstrates that curcumol induces senescent phenotypic changes in HepG2 cells, accompanied by G0/G1 phase cell cycle arrest.

Using quantitative real-time PCR, we analyzed the differential expression profiles of 81 senescence-related genes in curcumol-treated cells. The results revealed significantly elevated expression of TP53 and its downstream genes p16Ink4a, p21Waf1/Cip1, and p27Kip1, along with enhanced transcription of multiple senescence signaling pathway initiators and effectors including ABL1, ALDH1A3, CHEK2, HRAS, and PTEN. Conversely, expression of negative regulators of cell cycle progression and senescence signaling pathways, such as Cyclin A2, IGFBP3, SIRT1, and TERT, was significantly reduced. Western blot analysis confirmed increased protein levels of p53 and its downstream cyclin-dependent kinase inhibitors (CKIs) p21WAF1 and p16INK4, decreased Cyclin A2 levels consistent with PCR results, and significantly elevated wild-type p53-induced protein phosphatase 1 (Wip1). These findings suggest that curcumol induces senescence in HepG2 cells, likely through activation of

the p53 signaling pathway. This study provides valuable information for further investigation into the antitumor mechanisms of curcumol and its potential clinical applications.

Keywords: curcumol, premature senescence of tumor cells, HepG2 cells, liver cancer, cell cycle arrest

Introduction

Cellular senescence is an irreversible process in which cells transition from active proliferation to growth arrest. Senescent cells retain basic metabolic activity but lose DNA synthesis capacity and responsiveness to mitogenic stimulation. They exhibit increased lipofuscin deposition, enlarged and flattened morphology, organelle deformation, increased membrane fragility, nuclear membrane invagination, and express -galactosidase with high enzymatic activity at pH 6.0. Historically, tumor cells were considered incapable of senescence due to their unlimited proliferative potential. However, over the past decade, studies have shown that tumor cells can be induced into a senescent state under certain conditions, such as treatment with low-dose chemotherapeutic drugs or irradiation. This induced senescence, distinct from replicative senescence, is termed premature or accelerated senescence (Nardella et al, 2011). Research aimed at identifying drugs or molecular targets that can induce tumor cell senescence to restore and activate senescence pathways as a novel cancer therapeutic strategy has gained increasing attention (Nardella et al, 2011; Schosserer et al, 2017).

Curcumol (C H O), also known as curcumol, is a monomeric compound isolated from the traditional Chinese herb *Curcuma zedoaria* that inhibits proliferation of various human tumor cells (Wang et al, 2009; Lu et al, 2012; Huang et al, 2017). We previously reported that curcumol inhibits proliferation of human hepatocellular carcinoma HepG2 cells and significantly affects cell cycle progression and related regulatory gene expression (Huang et al, 2013). The present study further demonstrates that low-dose curcumol induces senescent phenotypic changes in HepG2 cells and provides preliminary analysis of differential expression profiles of 81 senescence-related genes and major regulatory molecules, offering valuable information for further exploration of curcumol' s antitumor mechanisms and potential clinical applications.

1.1 Reagents and Instruments

Curcumol (purity 98%, Shanghai Aihui Biotech, batch No. P02-03) was dissolved in absolute ethanol at 50 $mg \cdot ml^{1}$ for storage. Propidium iodide (PI) and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA). MTT was obtained from Amresco (USA). Rabbit anti-human p53 polyclonal antibody and mouse anti-human p16, Wip1, p21, and -actin monoclonal antibodies were from

Santa Cruz (USA). Instruments included a FACSAria III flow cytometer (BD, USA), iMark microplate reader (Bio-Rad, USA), and CO cell incubator (NBS, USA).

1.2 Cell Line and Culture

Human hepatocellular carcinoma HepG2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai) and routinely cultured in DMEM medium (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA) at 37 °C in a 5% CO incubator. Medium was changed and cells were passaged every 3–4 days.

2.1 MTT Assay for Cell Proliferation

HepG2 cells were seeded in 96-well plates at 4×10^3 cells per well and cultured overnight. The medium was replaced with curcumol-containing solutions at various concentrations (all containing equal volumes of ethanol vehicle) at 100 l per well. After incubation for predetermined times, 10 l of MTT was added per well and incubated at 37 °C for 4 h. The supernatant was removed, 200 l of DMSO was added per well, and plates were shaken in the dark for 20 min. Absorbance at 490 nm (OD) was measured using a microplate reader. Experiments were repeated three times. Cell growth inhibition rate was calculated as: Inhibition rate (%) = [1 - (OD d / OD)] × 100. IC values were calculated using SPSS software (version 20.0).

2.2 Flow Cytometry for Cell Cycle Analysis

HepG2 cells were cultured in serum-free DMEM for 5 days to synchronize most cells in G1 phase. Synchronized cells were seeded in 60 mm dishes at 5×10 cells per dish. After 20 h, the medium was replaced with curcumol-containing solutions at various concentrations (all containing equal volumes of ethanol). Cells were collected at 16 h, 24 h, and 32 h by routine digestion, fixed in 70% ethanol, treated with 1% RNase, stained with PI, and analyzed by flow cytometry for cell cycle distribution (DNA content). Experiments were repeated three times.

2.3 Senescence-Associated -Galactosidase (SA- -gal) Activity Assay

Cells were seeded in 6-well plates and treated with curcumol for 24 h. On days 7 and 10, the culture medium was removed, cells were washed once with PBS, fixed with 0.2% glutaraldehyde for 5 min, washed three times with PBS, and incubated with X-gal staining solution (150 mmol·L ^{1} NaCl, 1 mg·ml ^{1} X-gal, 5 mmol·L ^{1} K Fe(CN) , 2 mmol·L ^{1} MgCl , 40 mmol·L ^{1} NaPi, pH 6.0, 5 mmol·L ^{1} K Fe(CN)) at 37 °C for 6–24 h. Blue-stained positive cells were counted under a microscope in random fields.

2.4 Real-Time Quantitative PCR for Cell Cycle Regulatory Genes

Based on the list of 84 genes in the Human Cellular Senescence PCR Array from SABiosciences (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-050Z.html), gene-specific quantitative PCR primers were designed and synthesized by Invitrogen (Shanghai, USA). Real-time quantitative PCR (Sybr Green method) was performed to detect gene expression levels as previously described (Yang et al, 2015). Results were automatically calculated from standard curves by software. A total of 82 genes were successfully amplified (gene names and primer sequences are shown in Table 1). GAPDH and ACTB genes were used as internal references for normalization.

2.5 Western Blotting

Cells were collected and lysed on ice with RIPA buffer (0.5% sodium deoxycholate, 1.0% NP-40, 150 mmol · L ^{1} NaCl, 0.1% SDS, 50 mmol · L ^{1} Tris-pH 8.0, 0.2 mg · L ^{1} aprotinin, 1 mmol · L ^{1} PMSF, 0.5 mg · L ^{1} leupeptin). After centrifugation, the supernatant was collected, loading buffer was added, and samples were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membranes using a semi-dry method. Membranes were blocked with 5% skim milk at room temperature for 1 h, incubated with primary antibodies at 4 °C overnight, washed three times with TBS-T, incubated with HRP-conjugated secondary antibodies at room temperature for 1 h, and detected using ECL chemiluminescence.

2.6 Statistical Analysis

SPSS 20.0 statistical software was used for repeated measures ANOVA. Pairwise comparisons between samples were performed using LSD-t tests.

Results

3.1 Effects of Curcumol on HepG2 Cell Proliferation and Cell Cycle

HepG2 cells were treated with various concentrations of curcumol (0–1000.0 mg · L ^{1}) for 48 h and 72 h. Cell viability was measured by MTT assay to calculate IC values. As shown in Figure 1 [Figure 1: see original paper]A, curcumol significantly inhibited HepG2 cell proliferation in a time- and dose-dependent manner, with IC values of 529.5 mg · L ^{1} at 48 h and 173.5 mg · L ^{1} at 72 h.

To further examine the long-term effects of curcumol on HepG2 cell proliferation, cells were first synchronized in serum-free medium, then treated with 25 mg · L ^{1} curcumol for 24 h. The medium was replaced with curcumol-free medium, and cells were passaged at a 1:4 dilution when they reached 80% confluence on day 3. Cells treated with curcumol for 24 h, 72 h, and 120 h were collected, fixed in ethanol, stained with propidium iodide (PI), and analyzed by

flow cytometry. As shown in Figures 1B and 1C, compared with the control group, the percentage of G0/G1 phase cells increased from (62.88 ± 4.71)% and (66.13 ± 0.83)% to (69.17 ± 4.44)% and (84.31 ± 2.15)% after 24 h and 72 h of curcumol treatment, respectively, while S and G2 phase cells decreased correspondingly. At 120 h post-treatment, G1/G0 phase cells remained at (85.74 ± 5.30)% compared to (65.76 ± 1.52)% in the control group, with statistically significant differences. These results indicate that low-dose curcumol can induce prolonged G0/G1 phase arrest in HepG2 cells.

3.2 Curcumol Induces Senescence in HepG2 Cells

Prolonged cell cycle arrest may reflect a cellular senescence response. Senescent cells exhibit enlarged, flattened morphology, nuclear membrane invagination, organelle deformation, and positive SA- -gal staining. To determine whether curcumol-treated HepG2 cells undergo senescent phenotypic changes, we performed SA- -gal staining on days 7 and 10 following curcumol treatment (25 $mg \cdot L^{1}$) as described in section 3.1. Microscopic examination revealed that curcumol-treated cells displayed enlarged, flattened morphology. The percentages of blue-stained cells were (70.03 ± 8.37)% and (87.23 ± 6.89)% on days 7 and 10, respectively. In contrast, vehicle control cells showed no significant morphological changes, with SA- -gal positive rates of (2.28 ± 0.73)% to (4.40 ± 1.26)%. The difference between groups was statistically significant ($P < 0.01$, Figure 2 [Figure 2: see original paper]).

3.3 Effects of Curcumol on Senescence-Related Gene Expression Profiles in HepG2 Cells

To further investigate the mechanism of curcumol-induced senescence, we used Sybr Green real-time RT-PCR to screen differential expression of 81 senescence-related genes in curcumol-treated HepG2 cells, with ACTB as the normalization control. Compared with the vehicle control, treatment with 25 $mg \cdot L^{1}$ curcumol for 24 h significantly upregulated 20 genes—including ABL1, ALDH1A3, ATM, B2M, CALR, Cyclin D1, CD44, CDK2, p21-Cip1, p27-Kip1, p16-Ink4A, p19-Ink4D, TP53, COL3A1, ETS1, HRAS, IFNG, PRLP0, and PTEN—and significantly downregulated 8 genes, including Cyclin A2, GADD45A, HPRT1, IGFBP3, MORC3, SIRT1, TERT, and THBS1 (Figure 3 [Figure 3: see original paper], Table 2).

3.4 Western Blot Detection of p53 Protein Function

Finally, we validated the expression levels of p53 and its downstream cyclin-dependent kinase inhibitors (CKIs) by Western blotting. The results showed that curcumol treatment significantly increased protein levels of p53, p21WAF1, and p16INK4 in HepG2 cells, while decreasing Cyclin A2 levels—consistent with the downregulation of Cyclin A2 mRNA observed in PCR experiments. Additionally, wild-type p53-induced protein phosphatase 1 (Wip1) was significantly elevated. Combined with the transcriptional activation of p21WAF1, these

findings suggest that p53 activation may be a critical mechanism underlying curcumol-induced senescence in HepG2 cells (Figure 4 [Figure 4: see original paper]).

Discussion

Curcumol is a monomeric compound isolated from the traditional Chinese herb *Curcuma zedoaria*, with Guangxi being one of China' s major production regions. Both in vitro cell culture and animal experiments have demonstrated that curcumol inhibits proliferation and growth of various tumor cells, including liver, lung, gastric, ovarian, and nasopharyngeal cancers. Its mechanisms may involve induction of cell cycle arrest and apoptosis, inhibition of nucleic acid metabolism, suppression of tumor angiogenesis, and promotion of cell differentiation (Wang et al, 2009; Tang et al, 2015). However, the antitumor mechanisms of curcumol remain incompletely understood. We previously reported that curcumol significantly inhibits proliferation of human hepatocellular carcinoma HepG2 cells in a time- and dose-dependent manner, an effect associated with activation of the p53/pRB pathway, inhibition of Cyclin A expression, and upregulation of p21WAF1, p27KIP1, and CDK8 (Huang et al, 2013). Additionally, curcumol can induce apoptosis in p53-mutant triple-negative breast cancer MDA-MB-231 cells via activation of the p73-PUMA signaling pathway (Huang et al, 2017). In this study, we found that treatment with a relatively low dose (25 $mg \cdot L^{1}$) of curcumol induced G0/G1 phase arrest in HepG2 cells, accompanied by senescent phenotypic changes including enlarged, flattened morphology and expression of -galactosidase with high enzymatic activity at pH 6.0, indicating that curcumol possesses the biological activity to induce premature senescence in HepG2 cells.

Senescent cells are characterized by irreversible cell cycle arrest. In mammals, cell cycle progression is regulated at three checkpoints: the G1/S transition, G2/M transition, and spindle assembly checkpoint, under strict control by cyclins (Cyclins), cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs). CDKs are activated by forming complexes with their respective cyclins to drive cell cycle progression. For example, Cyclin A1 can bind to CDK2; knockout of Cyclin A1 leads to G1 phase arrest, while Cyclin A2 can bind to CDK1 or CDK2 during S phase to regulate G2/M transition (Pagano et al, 1992). Wu et al. reported that the plant extract tylophorine induces G0/G1 arrest in HepG2 cells by inhibiting Cyclin A2 expression, and that overexpression of Cyclin A2 can reverse this arrest (Wu et al, 2009). On the other hand, Cyclin-CDK complexes are directly regulated by CKIs, which are divided into two families: INK4 and CIP/KIP. The INK4 family primarily sequesters CDKs to prevent complex formation with cyclins, while the CIP/KIP family directly binds to Cyclin-CDK complexes to inhibit CDK activity and negatively regulate cell cycle progression.

In this study, high-throughput quantitative PCR analysis of 81 senescence-

related genes in curcumol-treated HepG2 cells revealed significantly increased expression of TP53 and its downstream genes p16Ink4a, p21Waf1/Cip1, and p27Kip1, along with enhanced transcription of senescence pathway initiators and effectors such as ABL1, ALDH1A3, CHEK2, HRAS, and PTEN. Conversely, expression of negative regulators including Cyclin A2, IGFBP3, SIRT1, and TERT was significantly decreased. It is generally accepted that when DNA damage occurs, p53 is activated to inhibit CDK2 and CDK3 activity via p21 and/or p27, or to activate p16 to block CDK4 and CDK6 binding to cyclins, thereby inhibiting the RB signaling pathway and arresting cells in G1/G0 or G2/M phase (Terzi et al, 2016; Gire and Dulic, 2015). The screening results showing upregulation of 20 genes and downregulation of 8 genes suggest that curcumol-induced senescence involves complex signal transduction mechanisms. In addition to confirming increased p16 and decreased Cyclin A2 levels, Western blot analysis revealed significantly elevated levels of p53 protein and its transcriptionally activated target p21WAF1, as well as wild-type p53-induced protein phosphatase 1 (Wip1), suggesting that p53 activation may be an important mechanism underlying curcumol-induced senescence in HepG2 cells.

In conclusion, this study demonstrates that curcumol can induce premature senescence in hepatocellular carcinoma HepG2 cells in vitro, likely through activation of the tumor suppressor gene p53, upregulation of CKI molecules including p21WAF1 and p16INK4, increased PTEN expression, and involvement of various senescence pathway-associated molecules, ultimately leading to G0/G1 phase cell cycle arrest. These findings provide valuable information for further investigation of curcumol' s antitumor mechanisms and its potential clinical applications.

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Note: Figure translations are in progress. See original paper for figures.