RESEARCH ARTICLE

Curcumin Inhibits MHCC97H Liver Cancer Cells by Activating ROS/TLR-4/Caspase Signaling Pathway

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Abstract

Curcumin can inhibit proliferation of liver cancer cells by inducing apoptosis, but the specific signaling pathways involved are not completely clear. Here, we report that curcumin inhibited proliferation of MHCC97H liver cancer cells by induction of apoptosis in a concentration dependent manner via stimulating intracellular reactive oxygen species (ROS) generation. Also, we showed that increased intracellular ROS formation activated the TLR-4/MyD-88 signaling pathway, resulting in activation of caspase-8 and caspase-3, which eventually led to apoptosis in MHCC97H cells. These results showed that as a prooxidant, curcumin exerts anti-cancer effects by inducing apoptosis via the TLR-4/MyD-88 signaling pathway.

Keywords: Curcumin - liver cancer - reactive oxygen species - toll-like receptor 4 - apoptosis

Introduction

In worldwide, especially in Asia and Africa, as a prevalent cancer, hepatocellular carcinoma (HCC) is one of the most common causes of mortality in patients with malignant diseases (Parkin et al., 2005). Due to lack of sufficient and effective therapeutic drugs in treatment of HCC, the prognosis is often poor in patients with advanced liver cancer (Torimura et al, 2013). Inducing apoptosis is considered an effective strategy in eliminating targeted cancerous cells (Debatin, 2004). There are two major pathways that conducting apoptotic signaling, namely the mitochondria- dependent pathway and death receptor- dependent pathway (Gupta et al., 2003). Different anticancer agents affect various target molecules, such as tumor- necrosis factor (TNF)- ligand superfamily member 10 (TNFSF10), Fas- associated DD adapter protein (FADD) and death inducing signaling complex (DISC) which belong to the death receptor- dependent apoptotic pathway (Hengartner et al., 2000); cytochrome c (Cyt c), direct inhibitor of apoptosis- binding protein (SMAC/DIABLO), apoptosis- inducing factor (AIF) and ednonuclease G (EndoG) which participate in the mitochondria- dependent apoptotic pathway (Tan et al., 2009). Both of the two signaling pathways trigger apoptosis eventually by activating caspase cascade signaling.

Toll- like receptors (TLRs), located on cell membrane (TLR-1, 2, 4, 5 and 6) and in cytoplasm (TLR-3, 7, 8 and 9), are important innate immune receptors, recognized as pathogen- associated molecular patterns, taking responsibility for transcription of several immune genes during immune responses to infection (Kawai, et al.,

2007). It is now suggested that the expression of TLR-4 is not only found in immune cells, but also identified in many kinds of tumor cells. Notably, TLR-4 signaling is involved in cancer development and progression (Kelly et al., 2006). It was reported that activation of TLR-4 signaling suppressed the proliferative capacity of cancer cells (Andreani et al., 2007). Considering that TLR-4 is linked directly to cell apoptosis by stimulating cleavage of caspase-8 (Han et al., 2004), and ROS was supposed to be one of the causes of up-regulating TLR-4 expression (Yu et al., 2008), TLR-4 induced apoptosis could be one of the potential mechanisms which merit further investigation.

Curcumin, also known as 1, 7- bis (4-hydroxy-3methoxyphenol)- 1, 6-heptadiene-3, 5-dione, isobtained and purified from plant called turmeric (Curcuma longa L.). Several previous studies proved the anti- cancer ability of curcumin was related to apoptosis induction (Khar et al., 1999; Aggarwal et al., 2003). The cytotoxicity against tumor cells depend on the curcumin's capacity of generating superoxide intracellularly (Morin et al., 2003). Though several theories could interpret the mechanism (s) of the cytotoxicity/apoptosis of curcumin, the link is still not completely elucidated. Thus, whether TLR-4 signaling induced apoptosis is responsible for the cytotoxicity of curcumin against cancer arouses our interest.

In order to know the role of curcumin in treatment of cancer better, we examined the activation of TLR-4 apoptotic signaling pathway in curcumin treated MCC97H cells in the present study. The results would not only benefit the study of the mechanism of anticancer property of curcumin, but also provide clues in seeking appropriate molecular target in treatment of liver cancer.

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Materials and Methods

Cell line and cell culture

Human HCC cell line MHCC97H (Liver Cancer Institute of Fudan University) was utilized in this study. The cells were cultured in 75 cm^2 cell culture flasks (BD) in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin (Gibco), 100μg/ml streptomycin (Gibco), 2 mmol/L gluatmine (Sigma). Cells were cultured in a humidified environment with 5% CO_2 and 95% fresh air.

Cell viability assessment

In this study, cell viability was assessed by colorimetric 3- (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay in accordance with the protocols described in previous studies (Chen et al., 2013). Briefly, equal number (2×10^4) of cells were planted in a 96-well culture plate (Corning), then treated by serially diluted curcumin (Sigma) at different concentrations (0, 15, 30 and 60 μmol/L) for 24 hours. After being washed by phosphate buffered saline (PBS) twice, cells were then incubated by MTT (Sigma) at final concentration of 5mg/ml for 4 hours. Then the cells were washed by PBS and dissolved by dimethyl sulfoxide (DMSO, Sigma). Absorbance value at 450nm was read by a 96-well plate reader (Bio-Rad). The inhibition rate was expressed as a ratio of number of nonviable cells in experimental wells (curcumin concentration at 15, 30 and 60 μmol/L) compare to that of control wells (curcumin concentration at 0 μmol/L).

Cell apoptosis examination

Cells at density of 5×10^3 per well were cultured in a 12- well culture plate (Corning). Serially diluted curcumin at different final concentrations (0, 15, 30 and 60 μmol/L) were added to incubate the cells for 24 hours. The apoptosis was then analyzed by flow cytometry using Annexin V-FITC (Santa Cruz) and propidiumiodine (PI, Santa Curz) double staining with a flow cytometer (FACS Calibur, BD) in accordance with the protocol described in previous study (Liu et al., 2013).

Intracellular reactive oxygen species (ROS) detection

Intracellular ROS generation was detected by 2, 7- dichlorofluoresceindiacetate (DCFH-DA) staining. After incubated with curcumin at different final concentrations (0, 15, 30 and 60 μmol/L) for 24 hours, cells were then incubated with 10μmol/L of DCFH-DA (Molecular Probes) at 37°C for 30 minutes in a dark compartment. A 488nm laser was used to excite the cells to generate florescence of 2, 7- dichlorofluorescein (DCF) at 530nm. Then the fluorescence was detected and analyzed by a flow cytometer (FACS Calibur, BD).

Real- time Polymerase Chain Reaction assay

Real- time polymerase chain reaction (RT-PCR) assay was conducted to examine the mRNA levels of TLR-4 and its downstream molecule myeloid differentiation factor 88 (Myd-88). Total RNA was extracted from cells incubated

Table 1. Primers for RT-PCR

Gene	Sequence	Size(bp)
TLR-4	Forward 5'-AGACATCCAAAGGAATACTGCAA-3'	180
	Reverse 5'-GCCTTCATGTCTATAGGTGATGC-3'	
MyD-88	Forward 5'-CTCCATTCCTCCTCCAGACACT-3'	196
	Reverse 5'-AAGGAGAGGAGTTGGTTC-3'	
GAPDH	Forward 5'-CATCACTGCCACTCAGAAGA-3'	330
	Reverse 5'-TGAAGTCACAGGAGACAACC-3'	

by curcumin at different final concentrations (0, 15, 30 and 60 μmol/L) by using TRI Reagent RNA Isolation Reagent (Sigma). cDNA was then synthesized by using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative analysis was performed by using SYBR Premix Ex TaqTM II (TaKaRa). Specific primers of TLR-4 and Myd-88 (Lv et al., 2009) for amplification were designed and synthesized by Genescript (Table 1). Relative gene copy number for TLR-4 and Myd-88 was performed by Δcycle threshold method, expression of GAPDH mRNA was introduced as the endogenous reference gene.

Western Blotting

After incubated with curcumin at different final concentrations (0, 15, 30 and 60 μmol/L) for 24 hours, 1×10^6 cells were washed by PBS and then suspended in 250μL lysis buffer (pH=7.5, 1% Triton X-100, 40mmol/L Tris-HCl, 150mmol/L KCl, 1 mmol/L EDTA, 100mmol/L NaVO3 and 1mmol/L PMSF). After protein extraction and concentration detection (BCA method), 50μg protein were separated by sodium dodecyl sulfate- poly-acrylamide gel electrophoresis (SDS- PAGE) and then transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore). The membranes were then incubated with antibodies against TLR-4 (1:1000, Abcam), Myd-88 (1:1000, Abcam), caspase-8 P18 (1:1000, Santa Cruz), caspase-3 P17 (1:1000, Santa Cruz) and GAPDH (1:1000, Santa Cruz) in TBST containing 5% defatted milk at 4°C for 12 hours. After washing, a horseradish peroxidase IgG antibody (Santa Cruz) was used to incubate the membrane at room temperature for 1 hour. The immunoblots on membrane were developed and exposed by using Western Blotting Luminal Reagent (Santa Cruz). Quantification of immunoblots' intensity were performed by Image J (GE Healthcare).

Statistical Analysis

All assays in this study were repeated independently three times. Data are expressed in a mean±SD manner. Differences between means were analyzed by Student's t-test. $p<0.05$ was considered to be statistically significant.

Results

Curcumin inhibits the proliferation of MHCC97H cells

MTT assay was used to examine curcumin's effect on proliferation of MHCC97H cells. The ratio of survival MHCC97H cells was shown in Figure 1B after incubated with curcumin at different concentrations (0, 15, 30 and 60 μmol/L). At concentration of 15, 30 and 60 μmol/L, curcumin exerted significantly obvious anti-proliferative effects.
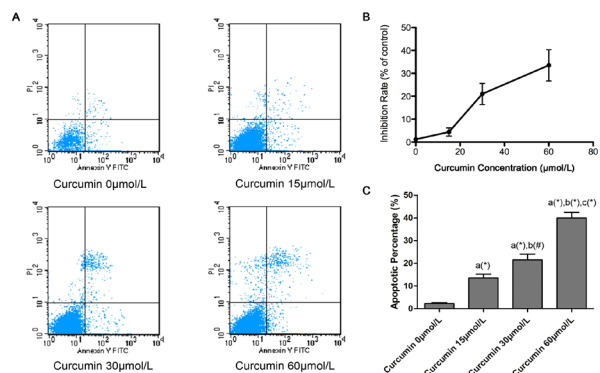
Figure 1. Effects of Curcumin on Proliferation and Apoptosis of MHCC97H Cells. A. Flow cytometry charts of annexin V and PI double staining in MHCC97H cells treated by curcumin at different concentrations. B. Inhibitory effects of curcumin on proliferation of MHCC97H cells was assessed by MTT assay. Line chart shows the inhibition rate of in MHCC97H cells incubated with curcumin at different concentrations. Values are represented in a (means±SD) manner. C. Columns represents the apoptotic percentage in MHCC97H cells incubated with curcumin corresponding to A. Values are represented in a (means±SD) manner. a Values are significantly different from 0μmol/L; b Values are significantly different from 15μmol/L; c Values are significantly different from 30 μmol/L [#*p*<0.05; **p*<0.01; &*p*<0.001]
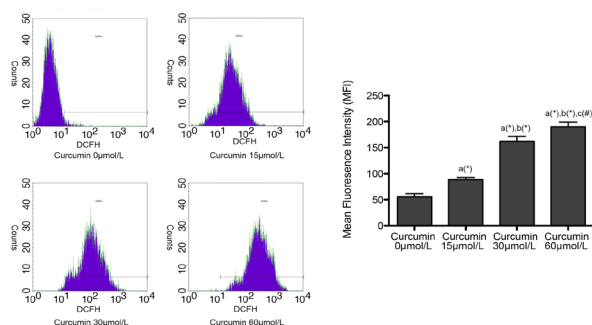

Figure 2. Intracellular ROS Generation in MHCC97H Induced by Curcumin Incubation. Left part of this figure shows the determination of ROS generation in MHCC97H cells incubated by curcumin at different concentrations. Cells were stained by DCFH-DA, followed by flow cytometry analysis. Columns at the right part of this figure represent the mean fluorescence intensity (MFI) in MHCC97H cells incubated by curcumin at different concentrations. Data is presented in a (means±SD) manner. a Values are significantly different from 0μmol/L; b Values are significantly different from 15μmol/L; c Values are significantly different from 30 μmol/L [#*p*<0.05; **p*<0.01; &*p*<0.001]

Curcumin induces the apoptosis of MHCC97H cells

Apoptosis of MHCC97H cells incubated with curcumin at different concentrations (0, 15, 30 and 60 μmol/L) was assessed by annexin V and PI double staining with a flow cytometer. Cell apoptosis was in a concentration dependent manner as shown in Figure 1A and 1C. The apoptotic cell population increased significantly after incubated with curcumin at concentrations of 15, 30 and 60μmol/L.

Curcumin evokes the intracellular ROS generation in MHCC97H cells

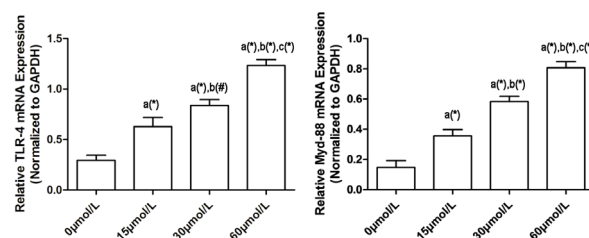The fluorescence released from DCF, which is the

Figure 3. mRNA Expression Levels of TLR-4 and MyD-88 in MHCC97H Cells Treated by Curcumin Incubation. Columns stand for the relative expression of TLR-4 and MyD-88 mRNA in MHCC97H cells incubated with curcumin at different concentrations. Values are represented in a (means±SD) manner. a Values are significantly different from 0μmol/L; b Values are significantly different from 15μmol/L; c Values are significantly different from 30 μmol/L [#*p*<0.05; **p*<0.01; &*p*<0.001]
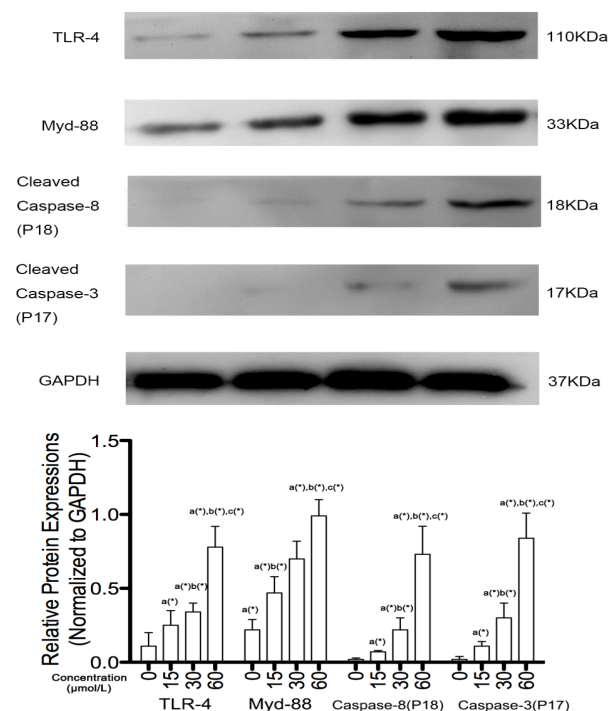

Figure 4. Effects of Curcumin on Protein Expression of TLR-4, MyD-88, Cleaved Caspase-8 and Cleaved Caspase-3 in MHCC97H Cells. The upper part of this figure shows the detection of immunoblots of TLR-4, MyD-88, caspase-8 P18, caspase-3 P17 and GAPDH protein expression levels by western blotting in MHCC97H cells incubated by curcumin at different concentrations. Quantification of the immunoblots is demonstrated by columns in the lower part of this figure. Data is presented in a (means±SD) manner. a Values are significantly different from 0μmol/L; b Values are significantly different from 15 μmol/L; c Values are significantly different from 30 μmol/L [#*p*<0.05; **p*<0.01; &*p*<0.001]

oxidized form of DCFH-DA could reflex the ROS generation level. Fluorescence intensity was detected by flow cytometry in MHCC97H cells incubated with curcumin at different concentrations (0, 15, 30 and 60 μmol/L). As shown in Figure 2, significant increase of DCF fluorescence intensity was detected in cells incubated with curcumin at different concentrations of 15, 30 and 60 μmol/L compared with 0 μmol/L respectively.

Curcumin upshifted mRNA expressions of TLR-4 and Myd-88 in MHCC97H cells

We used RT-PCR to investigate the promoting effect of curcumin on mRNA expression of TLR-4 and Myd-88 in MHCC97H cells. Cells were incubated with 0, 15, 30 and 60μmol/L curcumin for 24 hours and then subjected to RT-PCR. As demonstrated in Figure 3, we found that curcumin significantly increased expressions of TLR-4 and Myd-88 at transcriptional level in a concentration-dependent manner.

Curcumin upregulated TLR-4, Myd-88, cleaved caspase-8 and cleaved caspase-3 protein expressions in MHCC97H cells

Western blotting was utilized to assess the expressions of TLR-4, Myd-88, cleaved caspase-8 and cleaved caspase-3 at translational level. MHCC97H cells were treated by serial concentration of curcumin at 0, 15, 30 and 60 μmol/L for 24 hours before application of western blotting. As shown in Figure 4, treatment of curcumin significantly up- regulated protein expression of TLR-4, Myd-88, cleaved caspase-8 and cleaved caspase-3 in a concentration- dependent manner.

Discussion

As one of the most common malignant tumors, HCC is characterized by its fast progression and poor prognosis. It was reported that HCC is the third leading cause of cancer- related death (Caldwell et al., 2009), whose incidence is still growing fast worldwide (Venook et al., 2010). Though surgical operation is the main effective treatment option for HCC currently, assisted therapies such as chemotherapy and radiotherapy are still indispensable, especially for patients at advanced stage. However, the drug resistance and side effects of chemotherapy and radiotherapy become obstacles in achieving satisfied therapeutic effects. Thus, exploring effective anticancer drugs is of great significance.

Curcumin is a plant polyphenol extracted from spice turmeric (Curcuma longa), which is used as an herbal remedy in traditional Chinese and Indian medicine. In recent years, many studies verified the link between curcumin and numerous biological properties, such as anti-inflammatory, antioxidant, antiangiogenic, antidiabetic and anticancer activities (Hatcher et al., 2008; Liu et al., 2013). Curcumin exerts its anti- proliferative activity in various cancers including lung cancer, ovarian cancer, esophageal cancer, glioma and liver cancer (Kang et al., 2006; Pan et al., 2008; Wu et al., 2010) due to its capacity of inducing apoptosis (Karunagaran et al., 2005). Curcumin was also proved acting therapeutic efficacy against liver cancer in several previous investigations. It was suggested that curcumin inhibited proliferation of HepG2 liver cancer cells by inducing caspase-3 activity (Dai et al., 2013). In another study, Yu et al. proved that bax/bcl-2 signaling was involved in curcumin induced apoptosis of human hepatoma cells (Yu et al., 2011). It was reported that curcumin suppressed the expression of cyclin D1 to limit cell progression in cell cycle (Mukhopadhyay et al., 2002). Curcumin also could activate mitochondria-dependent apoptotic signaling pathway, leading to up- regulation of caspase cascade in tumor cell (Ahn et al., 2012). Curcumin's role of suppressing activation of NF-κB regulated antiapoptotic genes was also testified by previous study (Shakibaei et al., 2013). Though several anti-cancer mechanisms were indicated, we just reviled small part of the whole iceberg. Till currently, the specific pathways that link curcumin and its cytotoxicity of inducing apoptosis is still not completely elucidated. The further exploration would benefit the understanding and utility of curcumin- related anti-cancer drugs in patients with liver cancer.

Curcumin's role in influencing biological redox system seems complex and paradoxical because considerably evidences indicated that curcumin behaved as both antioxidant and proxiodant in vivo and in vitro (Iqbal et al.,2003; Atsumi et al.,2006). On one hand, it is proposed that the antioxidant property of curcumin is mediated by scavenging ROS (Mishra et al., 2004) and rebalancing endogenous redox system (Lopez-Lazaro et al.,2008). For instance, mice received dietary pretreatment of curcumin significantly inhibited ferric mitriloacetic acid- induced protein oxidation in the kidney (Iqbal et al., 2009). On the other hand, however, it is also well documented that curcumin acts as a prooxidant to incite intracellular ROS generation (Woo et al., 2003; Scott et al, 2004). For example, curcumin induced dramatic increase of ROS in small cell lung cancer NCI-H446 cell line (Yang et al., 2012). The association between ROS generation and curcumin- induced apoptosis in malignant cells has been already verified in previous investigations (Yoshino et al, 2004). The contradiction in curcumin's role of antioxidant and prooxidant could be reconciled by curcumin'sability to target malignant cells selectively (Zanotto-Fibno et al., 2012). Cancer cells are consistently under oxidative stress and more vulnerable to ROS- induced cytotoxity than normal cells (Martin-Cordero et al., 2012). Thus, curcumin at a specifically low concentration would induce apoptosis of malignant cells rather than normal ones. Similarly, in the present study, levels of ROS was found dramatically increased in MHCC97H cells treated by curcumin in a concentration- dependent manner.

TLRs consistent a receptor family which mediate recognition of pathogen- associated molecules, participating in innate immune responses (Janeway et al., 2002). TLR-4, which was the first described one among TLRs, whose expression was identified not only in immune cells, but also in many kinds of malignant cells (Qian et al., 2008). The role of TLR-4 in cancer seems discrepancy according to several previous studies. Huang and colleagues blocked TLR-4 signaling pathway by TLR-4 specific siRNA and blocking peptide in in tumor- bearing mice, the results turned out that the blocked delayed the growth of tumor and prolonged survival period of mice (Huang et al., 2005). However, opposite conclusions were drawn by several other studies. Simiantonaki et al. concluded that reduced TLR-4 expression is positively associated with metastasis of human colorectal cancer (Simiantonaki et al., 2007). Andereani and colleagues found that the activation of TLR-4 in tumor cells in vitro inhibited tumor growth in vivo (Andreani et al., 2007). In

brief, these studies focused on cancer immunity including inflammation, immune cell function, lymphocytes infiltration and so on when exploring related mechanism (s) and forming hypothesis. The effects of conducting TLR-4 signaling pathway in tumor cells themselves are rarely investigated.

It is reported that the expression of TLR-4 was regulated during oxidative stress in several studies (Powers et al., 2006; Dasu et al., 2008). On one hand, excessive intracellular ROS production resulted in up-regulation of TLR-4/2 expression (Huang et al., 2011). On the other hand, eliminating ROS by specific scavengers inhibited the up-regulation of TLR-4 expression (Matsuzawa et al., 2005) and suppressed transcription of TLR-4 mediated genes (Nakahira et al., 2006). Thus, ROS is thought as one of the causes to trigger the TLR-4 signaling pathway. Myd-88 is a down-stream adaptor molecule of TLR-4 to conduct signals to initiate translocation of nuclear factor kappa B (NF-$\varkappa$B) and activation of mitogen- activated protein kinases (MAPKs) which would eventually lead to cell apoptosis. It is believed that by interacting with Fas-associated death domian through Myd-88, caspase-8, the initiator of caspasecascade, could be activated by cleavage (Han et al., 2004). Activation of caspase-3, the executor of apoptosis in caspase cascade, could be activated also by cleavage after the activation of caspase-8 (Benchoua et al., 2002). In this study, TLR-4/Myd-88/Caspase-8/Caspase-3 pathway was found significantly activated after excessive ROS was generated by curcumin incubation in MHCC97H liver cancer cells. Thus, it could be proposed that curcumin induced intracellular generation of ROS stimulated expression of TLR-4. Then signals from TLR-4 activated its downstream molecules in this pathway, leading to cell apoptosis.

In short, curcumin exerted its anticancer effects on MHCC97H cells by inhibition of proliferation. Data collected in this study indicated that activation of ROS/TLR-4/Myd-88/Caspase-8/Caspase-8 could be a one of the novel mechanisms accounting for curcumin's cytotoxicity against MHCC97H cells.

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