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Curcumin regulates delta-like homolog 1 expression in activated hepatic stellate cell

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ABSTRACT

Hepatic stellate cell activation is a key cellular event in the development of liver fibrosis. Recently, Delta-like homolog 1 (DLK1) protein level has been shown to increase in HSC activation and serve as a new contributor to HSC activation and liver fibrosis. Curcumin, a natural yellow polyphenol, possesses therapeutic roles in many diseases including liver fibrosis and has long been used in traditional medicine. The present study was aimed to elucidate the effect of curcumin on DLK1 expression in HSCs in vitro and in vivo, which is still unknown. Our results demonstrated that curcumin reduced DLK1 expression in culture-activated HSCs and in rat model of liver fibrosis. The inhibitory effect of curcumin on DLK1 expression may be mediated in part by interruption of Shh signaling pathway, which contributes to the promotion effect of curcumin on the expression of PPAR-gamma, a key factor in inhibiting HSC activation. Our results in this study may reveal a new mechanisms through which curcumin exerts its inhibitory effect on HSC activation and liver fibrosis.

1. Introduction

Hepatic fibrosis is a reversible wound-healing response to either acute or chronic cellular injury. It is characterized by excessive production and deposition of extracellular matrix (ECM) molecules. Hepatic stellate cells (HSCs) has been established as the primary ECM-producing cell type during hepatic fibrogenesis (Lee and Friedman, 2011). In the normal liver, HSCs are in a quiescent state. Following chronic injury, quiescent HSCs transdifferentiate into activated myofibroblast-like cells (activated HSCs) and secret large amounts of ECM such as collagen. Therefore, HSC activation represents a key cellular event in the development of liver fibrosis (Hernandez-Gea and Friedman, 2011). HSCs can be activated by plating on the plastic, mimicking the process seen in vivo, which provides a good model for elucidating underlying mechanisms of HSC activation and studying potential therapeutic intervention of the process (Friedman, 2008).

Delta-like homolog 1 (DLK1/Pref-1) protein belongs to the epidermal growth factor (EGF) family of homeotic proteins and regulates several differentiation processes including inhibiting adipocyte differentiation (Garcés et al., 1999; Smas and Sul, 1993). Recently, it has been reported that DLK1 expresses selectively in HSCs in the adult rodent liver and is induced in liver fibrosis (Zhu et al., 2012). Furthermore, DLK1 expression is up-regulated in the process of HSC activation (Zhu et al., 2012). DLK1 knockdown in activated HSCs causes epigenetic derepression of peroxisome proliferator-activated receptor-gamma (PPAR-gamma), a key role in inhibiting HSC activation (Tsukamoto, 2005), and morphologic and functional reversal to quiescent cells (Zhu et al., 2012). DLK1 appears to serve as a new contributor to HSC activation and liver fibrosis (Pan et al., 2011).

Curcumin, an active polyphenol of the golden spice turmeric, is a highly pleiotropic molecule. Turmeric has been used for centuries as an anti-inflammatory remedy in Chinese medicine. Curcumin receives attention, because researches have found the roles of this polyphenol against cancer, pulmonary diseases, neurological diseases, liver diseases, metabolic diseases, autoimmune diseases, cardiovascular diseases, and numerous other chronic diseases (Gupta et al., 2013). Accumulating evidences demonstrate the inhibitory effect of curcumin on liver fibrosis. Curcumin can protect rat liver from CCl4-induced injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation (Fu et al., 2008). It suppresses HSC activation by inhibiting low-density lipoprotein receptorexpression (Kang and Chen, 2009) and leptin raising glucose levels (Tang and Chen, 2010a) and by up-regulation of PPAR-gamma expression (Zhou et al., 2007). Curcumin also inhibits leptin-induced HSC activation by accumulating intracellular lipids in HSCs (Tang and Chen, 2010b).

Therefore, it is Interesting to determine the relationship between curcumin and DLK1 expression in HSCs, which is still

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unknown. In the present studies, we elucidate the effect of curcumin on DLK1 in HSCs in vitro and in vivo.

2. Materials and methods

2.1. Materials

Curcumin was purchased from Sigma (St. Louis, MO, USA). Cyclopamine (a specific Hh signaling pathway antagonist of SMO) was purchased from selleck Chemicals (Houston, USA).

2.2. HSC isolation and culture

HSCs were isolated from Sprague-Dawley rats as we described previously (Zhou et al., 2010) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Rat HSCs between passages 3 and 6 were used for experiments. HSCs were treated with curcumin or vehicle dimethyl sulfoxide (without curcumin) in DMEM with 0.5%FBS.

2.3. Treatment of animals

Sprague-Dawley rats were randomly separated into 2 groups (six rats/each group) and respectively given administration of curcumin or phosphate buffered saline (PBS) throughout the 4-week period of thioacetamide (TAA) treatment. Curcumin (400 mg/kg body weight) or PBS (control) was given once daily by gavage (Fu et al., 2008) and TAA (200 μg/g body weight) was given by intraperitoneal injection two times a week (Yan et al., 2012). After 4-week, HSCs were isolated form the livers for Western blot analysis of DLK1 or DLK1 protein levels of HSCs were examined by double fluorescent staining of liver. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Nantong (2012-0031).

2.4. Western blot analysis

Western blot analysis was performed as we described previously (Zhou et al., 2010). Briefly, cells were lysed in ice-cold lysis buffer containing protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). After separated by SDS/PAGE and transferred to PVDF membrane, proteins were detected by primary antibodies against DLK1 (1:400), Sonic Hedgehog (Shh) (1:50), or β-actin (diluted 1:2000) and subsequently by horseradish peroxidase-conjugated secondary antibodies (diluted 1:4000). DLK1, Shh, and β-actin were purchased from Santa Cruz (Santa Cruz, CA, USA). β-Actin was used as an internal control. Representative immunoblots were shown from three independent experiments. The intensities of protein bands were densitometrically determined by Quantity One 4.4.1 (Bio-Rad Laboratories, Hercules, CA) and the graph showed the fold changes in the band densities relative to the control after normalization with the internal control.

2.5. Immunostaining of DLK1 and synaptophysin

Double fluorescent staining was used to examine the expression of DLK1 in HSCs in liver as we described previously (Zhou et al., 2010). Briefly, paraformaldehyde-fixed liver sections were blocked with normal serum and incubated with primary antibody against DLK1 (diluted 1:50, Santa Cruz, CA, USA) and primary antibody against synaptophysin (SYP, diluted 1:10, Abcam), a marker for quiescent and activated HSCs (Cassiman et al., 1999), followed by incubation with DyLight594-conjugated secondary antibody (diluted 1:500, ImmunoReagents, Inc. Raleigh, USA) and DyLight488-conjugated secondary antibody (diluted 1:500). The nuclei were counterstained with Hoechst 33342 (Sigma, St. Louis, MO, USA). The images were captured with the fluorescence microscope.

2.6. RNA isolation and real-time PCR

Total RNA was extracted using TRI-Reagent (Sigma, St. Louis, USA) according to the manufacturer's instructions. Real-time PCR was performed as we described previously (Zhou et al., 2009). Briefly, reverse transcription conditions were as follows: 42 °C for 15 min, 95 °C for 5 min, and 5 °C for 5 min. Real-time PCR was carried out in 25 μl of reaction solution and the reactions started at 95 °C for 7 min, followed by 40 cycles of 95 °C for 20 s, 54 °C for 30 s, and 72 °C for 30 s.

Fold changes in mRNA levels of target gene relative to the endogenous cyclophilin control were calculated as suggested by Schmittgen et al. (2000). Briefly, the cycle threshold (*Ct*) values of each target gene were subtracted from the *Ct* values of the housekeeping gene cyclophilin ($=\Delta Ct$). Target gene $\Delta\Delta Ct$ was calculated as ΔCt of Target gene minus ΔCt of control. The fold change in mRNA expression was calculated as $2^{-\Delta\Delta Ct}$. The primers used in real-time PCR were shown in Table 1.

2.7. Transient transfection

HSCs were cultured in 12-well plastic plates and transiently cotransfected with the DLK1 promoter (−1950/+36)-Luciferase construct (pGL3DLK1-Luc, a gift from Dr. Sang Hoon Kim, Kyung Hee University, Republic of Korea) (1.6 μg DNA/well,) plus 30 ng of control vector expressing Renilla Luciferase (pRL-TK; Promega, Madison, USA) by LipofectAMINE reagent (Life Technologies, New York, USA) and manufacturer's instructions. After transfection, HSCs were treated with different doses of curcumin. Luciferase activity was quantified fluorimetrically by using the Dual-Luciferase Reporter

Table 1
primers used in real-time PCR.

Rat gene	Primer
DLK1	(Forward) 5′-GGCCATCGTCTTTCTCAACA-3′ (Reverse) 5′-ATCCTCATCACCAGCCTCCT-3′
Shh	(Forward) 5′-CTGGCCAGATGTTTTCTGGT-3′ (Reverse) 5′-TAAAGGGGTCAGCTTTTTGG-3′
PPAR-gamma	(Forward) 5′-ATTCTGGCCCACCAACTTCGG-3′ (Reverse) 5′-TGGAAGCCTGATGCTTTATCCCCA-3′
α-Smooth muscle actin (α-SMA)	(Forward) 5′-ACAACGTGCCTATCTATGAGGGCT-3′ (Reverse) 5′-AGCGACATAGCACAGCTTCTCCTT-3′
α1(1) Procollagen	(Forward) 5′-TTCCCTGGACCTAAGGGTACT-3′ (Reverse) 5′-TTGAGCTCCAGCTTCGCC-3′
Cyclophilin	(Forward) 5′-TGGATGGCAAGCATGTGGTCTTTG-3′ (Reverse) 5′-CTTCTTGCTGGTCTTGCCATTCCT-3′