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Research Paper

# Curcumol induces RIPK1/RIPK3 complex-dependent necroptosis via JNK1/2-ROS signaling in hepatic stellate cells

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ABSTRACT

It is generally recognized that hepatic fibrogenesis is an end result of increased extracellular matrix (ECM) production from the activation and proliferation of hepatic stellate cells (HSCs). An in-depth understanding of the mechanisms of HSC necroptosis might provide a new therapeutic strategy for prevention and treatment of hepatic fibrosis. In this study, we attempted to investigate the effect of curcumol on necroptosis in HSCs, and further to explore the molecular mechanisms. We found that curcumol ameliorated the carbon tetrachloride ($CCl_4$)-induced mice liver fibrosis and suppressed HSC proliferation and activation, which was associated with regulating HSC necroptosis through increasing the phosphorylation of receptor-interacting protein kinase 1 (RIPK1), receptor-interacting protein kinase 3 (RIPK3). Moreover, curcumol promoted the migration of RIPK1 and RIPK3 into necrosome in HSCs. RIPK3 depletion impaired the anti-fibrotic effect of curcumol. Importantly, we showed that curcumol-induced RIPK3 up-regulation significantly increased mitochondrial reactive oxygen species (ROS) production and mitochondrial depolarization. ROS scavenger, N-acetyl-L-cysteine (NAC) impaired RIPK3-mediated necroptosis. In addition, our study also identified that the activation of c-Jun N-terminal kinase1/2 (JNK1/2) was regulated by RIPK3, which mediated curcumol-induced ROS production. Down-regulation of RIPK3 expression, using siRIPK3, markedly abrogated JNK1/2 expression. The use of specific JNK1/2 inhibitor (SP600125) resulted in the suppression of curcumol-induced ROS production and mitochondrial depolarization, which in turn, contributed to the inhibition of curcumol-triggered necroptosis. In summary, our study results reveal the molecular mechanism of curcumol-induced HSC necroptosis, and suggest a potential clinical use of curcumol-targeted RIPK1/RIPK3 complex-dependent necroptosis via JNK1/2-ROS signaling for the treatment of hepatic fibrosis.

## 1. Introduction

Hepatic fibrosis caused by multiple chronic liver injuries, is a known contributor to cirrhosis, and even liver cancer [1,2]. This scarring process starts with activation and proliferation of hepatic stellate cells (HSCs). Activated HSCs trans-differentiate into myofibroblasts during liver fibrosis, leading to the secretion and deposition of extracellular matrix (ECM) components [3,4]. A growing evidence has shown that hepatic fibrosis is reversible [5–7]. The elimination of activated HSCs through cell death, including apoptosis, senescence, autophagy has been regarded as an effective antifibrogenic strategy [8–10]. We previously reported that HSC senescence could enhance immune surveillance, inhibit ECM components production, and consequently improve liver fibrosis [11]. Our recent study showed that the inhibition of autophagy in activated HSCs restored lipocyte phenotype, which was beneficial for the reverse of hepatic fibrosis [12]. Recent studies have highlighted a new model of programmed cell death, necroptosis, which is closely involved in liver disease including hepatocellular carcinoma (HCC), alcoholic fatty liver disease, and non-alcoholic fatty liver disease [13–15]. Investigations on necroptosis in liver fibrosis, however, are

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rarely performed. Until recently, only one published study showed that gallic acid could trigger necroptosis in activated HSCs [16]. In the current study, we intend to evaluate the role of necroptosis in liver fibrosis and further to explore the underlying molecular mechanisms.

Necroptosis is characterized as the cell death with the similar morphology as necrosis and the unique upstream signal pathway just as apoptosis [17]. Necroptosis may serve as an alternate pathway to enable cell death when apoptosis is restrained. Receptor-interacting protein kinase 1 and 3 (RIPK1 and RIPK3) are regarded as central regulators for initiating necroptosis [18,19]. Activated RIPK1 binds to RIPK3, generating the necrosome complex. Necrosome could recruit and promote mixed lineage kinase domain-like (MLKL) phosphorylation [20]. Then, the activated MLKL oligomerizes and binds to membrane phospholipids, promoting the formation of pores that cause necroptotic cell death [21]. Recently, growing evidence has showed that reactive oxygen species (ROS) could change mitochondrial permeability, eventually leading to necroptosis [22]. However, it is still unknown whether the programmed necrosis ultimately result in cell death through the mitochondrial ROS pathway or the permeable pores induced by MLKL in some certain cells [23]. Moreover, the roles of RIPK1 and RIPK3 remain unclear in regulating ROS-mediated necroptosis. We previously reported that ROS-JNK1/2-induced autophagy in activated HSCs ameliorated inflammatory microenvironment [24]. It is interesting to explore whether ROS generation contributes to HSC necroptosis.

It is well-known that intracellular ROS could regulate mitogen activated protein kinases (MAPKs), including c-Jun N-terminal kinase1/2 (JNK1/2), extracellular regulated kinase1/2 (ERK1/2), and p38, which are the critical kinases that participate in numerous biological process, such as apoptosis, autophagy, and cell survival [25–27]. Meanwhile, ROS is vital for ferroptosis, a newly discovered type of regulated cell death [28]. Interestingly, recent study reported JNK activation could contribute to intracellular ROS production, promoting poly (ADP-ribose) polymerase-1 (PARP-1) dependent cell death (parthanatos) in glioma cells [29]. Besides, JNK could be phosphorylated by RIPK3, and activated JNK might contribute to necrosis via advancing the generation of intracellular ROS in hepatocytes [30]. These discoveries show that the JNK/ROS signaling pathway is important for cell survival. Thus, whether RIPK3/JNK/ROS signaling pathway involves in HSC necroptosis is worth further exploring.

Curcumol, a guaiane-type sesquiterpenoid hemiketal extracted from the roots of the herb *Rhizoma Curcumae*, exhibits multiple-pharmacological activities, including anti-inflammatory, and anti-tumor effect [31,32]. A previous study reported that curcumol induced HSC-T6 cell death [33], but no major research of curcumol on liver fibrosis has been done. In the present study, we are the first to evaluated the effect of curcumol on protecting the liver from carbon tetrachloride ($CCl_4$)-induced injury and fibrogenesis. Importantly, we verify that curcumol-induced RIPK1/RIPK3 complex promoted ROS production in HSCs via activating JNK1/2, thus triggering HSC necroptosis. Our study shows that curcumol can be a potential chemotherapeutic agent for the treatment of liver fibrosis.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Curcumol, N-acetyl cysteine (NAC), N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), necrotatin-1 (Nec-1), and SP600125 were brought from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), Opti MEM medium, phosphate buffered saline (PBS), and trypsin-EDTA were purchased from GIBCO BRL (Grand Island, NY, USA). Oxidation sensitive 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were obtained by Beyotime Biotechnology (Shanghai, China). MitoSox Red was brought from Thermo Fisher Scientific (Waltham, MA, USA). Anti-rabbit IgG, anti-mouse IgG and antibodies against β-actin, and RIPK1 (17519-1-AP) were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). Main antibodies against α-SMA, α1(I) procollagen, EGFR, fibronectin, MLKL, PDGF-βR, p-MLKL, p-RIPK3, RIPK3, and TGF-βRII were obtained from Abcam Technology (Abcam, Cambridge, UK). Primary antibodies against caspase3, cleaved-caspase3, caspase8, cleaved-caspase8, caspase9, cleaved-caspase9, ERK, JNK, p38, p-ERK, p-JNK, p-p38, p-RIPK1 and TIMP2 were procured by Cell Signaling Technology (Danvers, MA, USA). Lentivirus vectors encoding negative control shRNA (NC shRNA) and RIPK3 shRNA were designed by Hanbio (Shanghai, China).

### 2.2. Animal procedures and treatments

All experimental procedures received the approval of the institutional and local committee on the care and use of animals in Nanjing University of Chinese Medicine (Nanjing, China). The whole animals were given humane care according to the National Institutes of Health guidelines. Male ICR mice weighing about 18–22 g were purchased from Nanjing Medical University (Nanjing, China). A mixture of $CCl_4$ (0.1 ml/20 g body weight) and olive oil (1:9 (v/v)) was utilized to trigger hepatic fibrosis in mice by intraperitoneal injection. Male ICR mice were randomly divided into seven groups (eight mice per group). Mice in groups 1–7 were correspondingly given the following treatments: group 1, negative control (NC) shRNA lentivirus and olive oil; group 2, NC shRNA lentivirus, $CCl_4$, and olive oil; Mice in groups 3–5, NC shRNA lentivirus, $CCl_4$, olive oil, and curcumol with 15, 30 and 60 mg $kg^{-1}$, respectively, serving as treatment groups; group 6, RIPK3 shRNA lentivirus, $CCl_4$, and olive oil; group 7, RIPK3 shRNA lentivirus, $CCl_4$, olive oil and curcumol (30 mg $kg^{-1}$). Mice in groups 2–7 were intraperitoneally injected with $CCl_4$ every day for 8 weeks. Curcumol was dissolved in olive oil and given every other day via intraperitoneal injection during the 5–8 weeks period. Lentivirus with a titer of $2.5 \times 10^7$ pfu/g was injected into caudal vein of mice once per 2 weeks. At the end of the experiment, all mice were anesthetized with an injection of pentobarbital (50 mg $kg^{-1}$), and then sacrificed. Blood was gathered for biochemical indicators and ELISA assay. A partial liver was removed for histopathological, immunofluorescence, and immunohistochemical studies by fixing with 10% formalin and subsequently embedded with paraffin. The rest of liver was cut into pieces and immediately frozen with liquid nitrogen for extracting total RNA and proteins.

### 2.3. Liver histopathology and immunohistochemistry

Hematoxylin and eosin (H&E), Masson and Sirius Red staining were conducted according to the previously established methods. Immunohistochemical staining was performed using antibodies against CD45, F4/80, and α-SMA as previously described [11]. Photographs were taken in a blinded fashion at random fields. Representative pictures of liver sections were displayed.

### 2.4. Cell culture

Primary HSCs were isolated from male ICR mice (Nanjing Medical University, Nanjing, China) as previously described [29]. Human HSC-LX2 cell line was brought from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Isolated HSCs and HSC-LX2 cells were cultured in DMEM with 10% FBS, 1% antibiotics, and incubated in a 5% $CO_2$ and 95% air humidified atmosphere at 37 °C.

### 2.5. Cell viability assay

Cellular viability was detected by the Cell Counting Kit-8 (Beyotime Biotechnology, Shanghai, China). Cells were cultured in 96-well plates