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The inhibition of activated hepatic stellate cells proliferation by arctigenin through G_0/G_1 phase cell cycle arrest: Persistent $p27^{Kip1}$ induction by interfering with PI3K/Akt/FOXO3a signaling pathway

Ao Li [a,*,1], Jun Wang [a,1], Mingjun Wu [b,1], Xiaoxun Zhang [a], Hongzhi Zhang [a]

[a] College of Pharmacy and Bioengineering, Chongqing University of Technology, 69 Hong Guang Avenue, Chongqing 400054, PR China
[b] Institute of Life Sciences, Chongqing Medical University, 1 Yi Xue Yuan Road, Chongqing 400016, PR China

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ABSTRACT

Proliferation of hepatic stellate cells (HSCs) is vital for the development of fibrosis during liver injury. In this study, we describe that arctigenin (ATG), a major bioactive component of *Fructus Arctii*, exhibited selective cytotoxic activity *via* inhibiting platelet-derived growth factor-BB (PDGF-BB)-activated HSCs proliferation and arrested cell cycle at G_0/G_1 phase, which could not be observed in normal human hepatocytes *in vitro*. The cyclin-dependent kinase (CDK) 4/6 activities could be strongly inhibited by ATG through down-regulation of cyclin D1 and CDK4/6 expression in early G_1 phase arrest. In the ATG-treated HSCs, the expression level of $p27^{Kip1}$ and the formation of CDK2-$p27^{Kip1}$ complex were also increased. $p27^{Kip1}$ silencing significantly attenuated the effect of ATG, including cell cycle arrest and suppression of proliferation in activated HSCs. We also found that ATG suppressed PDGF-BB-induced phosphorylation of Akt and its downstream transcription factor Forkhead box O 3a (FOXO3a), decreased binding of FOXO3a to 14-3-3 protein, and stimulated nuclear translocation of FOXO3a in activated HSCs. Furthermore, knockdown of FOXO3a expression by FOXO3a siRNA attenuated ATG-induced up-regulation of $p27^{Kip1}$ in activated HSCs. All the above findings suggested that ATG could increase the levels of $p27^{Kip1}$ protein through inhibition of Akt and improvement of FOXO3a activity, in turn inhibited the CDK2 kinase activity, and eventually caused an overall inhibition of HSCs proliferation.

1. Introduction

Liver fibrosis, a common outcome of chronic hepatocellular damage, could be induced by a variety of etiological factors, including viral infections, hepatic toxins, alcohol abuse and autoimmune reactions and so on (Hui and Friedman, 2003). Liver fibrosis, whose pathological process is progressive, is characterized by increased deposition of extracellular matrix (ECM) proteins. Meanwhile, liver fibrosis is a key stage of disease process from hepatic damage to cirrhosis or even to hepatocellular carcinoma (Bataller and Brenner, 2005; Tsukada et al., 2006). Hepatic stellate cells (HSCs) have been considered as the primary source of ECM in liver injury (Friedman, 2010; Lotersztajn et al., 2005). According to the recent studies, the HSCs proliferation and expansion of their pool were a key step during the fibrogenic process (Son et al., 2013; Sun et al., 2009). The hepatic ECM accumulation resulted from both increased number and gene expression changes of activated HSCs. Hence, suppression of activated HSCs proliferation has been proposed as a therapeutic strategy for the treatment and prevention of liver fibrosis.

The cell cycle is a common convergent point for the mitogenic signaling cascades. In activated HSCs, every major checkpoint in

* Corresponding author. Tel.: +86 23 62400251; fax: +86 23 68906603.
E-mail address: ao_li@cqut.edu.cn (A. Li).
[1] These authors contributed equally to this work.

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cell cycle has been controlled by multiple protein kinases, each of which contains a regulatory cyclin component and a catalytic cyclin-dependent kinase (CDK) (Peng et al., 2013). The kinase activities of CDK–cyclin complexes would be inhibited by two classes of cyclin-dependent kinase inhibitors (CKIs). One is the INK4 protein family consisting of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, specifically inhibiting CDK4 and CDK6. The other is the kinase inhibiting protein (KIP) family consisting of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which can inhibit several CDKs (Owa et al., 2001). Specifically, $p27^{Kip1}$ could negatively regulate the activity of protein kinase complex cyclin E–CDK2, thereby blocking cell cycle progression from G_1 to S phase (Egozi et al., 2007). The aberrations in the G_1 regulation following liver injury often lead to rapid proliferation of HSCs. Upon mitogenic stimuli, especially platelet-derived growth factor-BB (PDGF-BB), the level of $p27^{Kip1}$ is down-regulated, allowing cyclin E–CDK2 to drive HSCs into the S phase and promote rapid proliferation (Kossatz et al., 2004).

In our pervious work, herbs were screened for inhibiting the proliferation of HSCs. An ethanol extract from *Fructus Arctii* was active in preventing cell growth of HSCs activated by PDGF-BB. As a main bioactive component from *Fructus Arctii*, arctigenin (ATG) has been commonly applied in the clinical practice for reducing inflammation in diverse contexts, including treatment of anemopyretic cold, sweeling of throat, cough, measles, and syphilis in China and other Asian countries (Cho et al., 2004; Lee et al., 2010). However, few studies have focused on the therapeutic or chemopreventive properties of ATG on hepatic fibrogenesis, both *in vitro* or *in vivo*. In the present study, we aimed to investigate the effects of ATG on the proliferation-related events of activated HSCs and to clarify the underlying molecular mechanisms.

2. Materials and methods

2.1. Materials

Arctigenin (ATG, > 99% purity; MW: 372.41) was purchased from Nanjing Zelang Medical Technology Co. Ltd. (Nanjing, China). A stock solution of ATG with the concentration of 16 mM was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C until use. The final concentration of DMSO in all experiments did not exceed 0.1% (v/v); this concentration did not cause any obvious deleterious effects on cell viability (data not shown). Antibodies against the following proteins were used: phospho-(Thr183/Tyr185)-stress-activated protein kinase (SAPK)/c-Jun NH2 kinase (JNK), phospho-(Thr202/Tyr204)-extracellular signal-regulated kinase (ERK)1/2, phospho-(Thr180/Tyr182)-p38 mitogen-activated protein kinase (MAPK), SAPK/JNK, ERK1/2, p38 MAPK (Cell Signaling Technology, Beverly, MA, USA); PDGF receptor-β (PDGFRβ) and phosphotyrosine (BD Biosciences, San Diego, CA, USA); phospho-(Thr308)-Akt, Akt, CDK2, CDK4, CDK6, and lamin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-(Ser473)-Akt, 14-3-3, cyclin D1, cyclin E1, $p21^{Cip1}$, $p27^{Kip1}$, Forkhead box O 3a (FOXO3a), FOXO1, FOXO3a, FOXO4, and phospho-(Ser262)-FOXO4 (Epitomics, Burlingame, CA, USA); Fluorescein isothiocyanate (FITC)- or horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd (Beijing, China). LY294002 (phosphoinositide 3-kinase inhibitor; PI3K inhibitor), SB203580 (p38 MAPK inhibitor), normal rabbit IgG, and primary antibodies for phospho-(Thr24)-FOXO1/(Thr32)-FOXO3a and β-actin were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). U0126 (inhibitor of MAPK kinase; inhibitor of MEK1/2) was provided by Cell Signaling Technology. PHT-427 (protein kinase B/Akt inhibitor; Sheng et al., 2011) was purchased from Selleck Chemicals (Houston, TX, USA). Propidium iodide (PI) and all the other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and treatment

LX-2 was a well-characterized human HSCs line. Many features of activated HSCs phenotype could be recapitulated in LX-2, including the expression of the PDGFRβ and proliferation in response to PDGF (Xu et al., 2005). LX-2 cells were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37 °C in humidified air with 5% CO_2. Human embryonic hepatocytes cell line L-02 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium (Hyclone). The culture conditions were the same as the conditions described above. The LX-2 and L-02 cells were prepared with passages between 18 and 25. Both of two cells were collected for application in relevant experiments. When cells were 70% confluent, they were cultured in medium without FBS for 24 h to synchronize, then the cells were pre-incubated for 4 h in medium containing 0.5% FBS and either DMSO (vehicle control) or ATG. After pre-incubation, human recombinant PDGF-BB (Peprotech, Rocky Hill, NJ, USA) with a final 50 ng/ml concentration was added to the culture medium without wash and the mixture was incubated for different time intervals at 37 °C until further assays.

2.3. Assay of cell proliferation

LX-2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well. The cell proliferation was analyzed with 5-bromo-2-deoxyuridine (BrdU)-based Cell Proliferation ELISA kit (Roche Applied Science, Mannheim, Germany). Briefly, cells were synchronized after serum starvation for 24 h. Then cells were transfected with either $p27^{Kip1}$-specific siRNA or scrambled control siRNA and/or pre-treated with ATG for certain time periods. All the cell groups were then exposed to 50 ng/ml PDGF-BB for 12, 24 or 48 h. In the last 4 h of treatment, the cells were labeled with 10 μM BrdU. After disposing the labeling medium, the genomic DNA of cells was fixed and denatured with FixDenat solution. The BrdU incorporated in the cells was detected by a peroxidase-conjugated anti-BrdU antibody and quantified by measuring the absorbance (*A*) value at 370 nm with a universal microplate autoreader (Varioskan, Thermo Electron Co., Waltham, MA, USA; the reference wavelength was 492 nm). Absorbance values were directly correlated with the amount of synthesized DNA, as well as indicating the number of proliferated cells. Each time point was conducted in triplicate and the experiment was repeated for six times. The cell proliferation inhibition rate (IR%) was calculated with the following formula:

$$IR\% = (A_{control} - A_{treated})/(A_{control} - A_{blank}) \times 100\%$$

where $A_{treated}$ was the *A* value in wells treated with ATG before the exposure of PDGF-BB stimulation, $A_{control}$ was the *A* value in wells treated with DMSO before the exposure of PDGF-BB stimulation, and A_{blank} was the *A* value in wells without cells but incubated with DMSO. The IC_{50} value was designated as the concentration that caused 50% inhibition of cells proliferation, and was calculated by SPSS 16.0 for windows (IBM, Chicago, IL, USA).

2.4. Assay of lactate dehydrogenase (LDH) release

L-02 or LX-2 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. The cells were incubated in RPMI 1640 or DMEM medium supplemented with 10% FBS for 24 h. Then cells were synchronized with serum starvation for another 24 h. After