Toxicology 326 (2014) 9–17

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Galangin suppresses HepG2 cell proliferation by activating the TGF-β receptor/Smad pathway

Yajun Wang [a,b], Jun Wu [b], Biyun Lin [a], Xv Li [a], Haitao Zhang [a,*], Hang Ding [a], Xiaoyi Chen [a], Liubo Lan [a], Hui Luo [c,**]

[a] Department of Biochemistry and Molecular Biology, Guangdong Medical College, Dongguan, Guangdong 523808, China
[b] Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong 524001, China
[c] Department of Chemistry, Guangdong Medical College, Zhanjiang, Guangdong 524023, China

ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form 21 September 2014
Accepted 22 September 2014
Available online 28 September 2014

Keywords:
Galangin
Autophagy
Smad
TGF-β receptor
Autophagy-related gene

ABSTRACT

Galangin can suppress hepatocellular carcinoma (HCC) cell proliferation. In this study, we demonstrated that galangin induced autophagy by activating the transforming growth factor (TGF)-β receptor/Smad pathway and increased TGF-β receptor I (RI), TGF-βRII, Smad1, Smad2, Smad3 and Smad4 levels but decreased Smad6 and Smad7 levels. Autophagy induced by galangin appears to depend on the TGF-β receptor/Smad signalling pathway because the down-regulation of Smad4 by siRNA or inhibition of TGF-β receptor activation by LY2109761 blocked galangin-induced autophagy. The down-regulation of Beclin1, autophagy-related gene (ATG) 16L, ATG12 and ATG3 restored HepG2 cell proliferation and prevented galangin-induced apoptosis. Our findings indicate a novel mechanism for galangin-induced autophagy via activation of the TGF-β receptor/Smad pathway. The induction of autophagy thus reflects the anti-proliferation effect of galangin on HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, especially in Asia. Chemotherapy is a common therapeutic strategy after surgery, but its use has been limited due to its toxicity to normal tissues. Natural products have long been used for anti-cancer treatment and are potentially safer alternatives (Surh, 2003).

Galangin is a polyphenolic compound with a molecular weight of 270.24 Da that is primarily derived from the rhizome of *Alpinia officinarum* Hance, which is used in traditional Chinese medicine and for dietary purposes (Heo et al., 2001). We have previously shown that galangin significantly decreases cell viability and induces apoptosis in HCC lines, and we proposed galangin as a potential anti-HCC agent (Zhang et al., 2012). Further, reports by Wen et al. (2012) and Zhang et al. (2013) have described galangin-induced autophagy in HepG2 cells. These observations suggest that galangin inhibits the proliferation of HepG2 cells by a novel mechanism. However, the precise mechanism by which galangin induces autophagy remains unclear.

Autophagy is an evolutionally conserved lysosomal degradation pathway. During autophagy, the isolation membrane envelops some cytoplasmic contents and then transforms into the autophagolysosome via fusion with the lysosome. The contents are then degraded by lysosomal enzymes (Mathew et al., 2007). Autophagy-related gene (ATG) proteins play essential roles in autophagy. Autophagosome formation is mediated by ATG protein systems, which alter the microtubule-associated protein 1 light chain 3 (LC3-I) to its phosphatidylethanolamine-conjugated membrane-bound form (LC3-II) via conjugation of the ATG5-ATG12 complex (Shibutani and Yoshimori, 2014).

Beclin1, also called ATG6/Vps30, plays an important role in initiating autophagosome formation. Beclin1 is a tumour suppressor gene because it is monoallelically deleted in 40% to 75% of human ovarian, prostate and breast cancers (Aita et al., 1999; Liang et al., 1999). Loss of Beclin1 may cause genomic instability, which ultimately initiates tumourigenesis (Mathew et al., 2007).

Transforming growth factor-β (TGF-β) superfamily signalling plays a critical role in the regulation of cell growth, differentiation, and migration and in the development of various cell types. Normally, signalling is initiated by the ligand-mediated oligomerisation of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signalling molecules Smad2 and Smad3 for the

* Corresponding author. Tel.: +86 769 22896339/7592388764; fax: +86 759 2388246.
** Co-corresponding author.
E-mail addresses: taohaizhang@tom.com, taohaizhang33@hotmail.com (H. Zhang), luohui@gdmc.edu.cn (H. Luo).

http://dx.doi.org/10.1016/j.tox.2014.09.010
0300-483X/

TGF-β pathway or Smad1/5/8 for the bone morphogenetic protein (BMP) pathway. TGF-β/Smad signalling acts as both a tumour suppressor and tumour promoter, similar to the duality of autophagy in oncogenesis (Bierie and Moses, 2006). Suzuki et al. (2010) reported that TGF-β/Smad signalling induces autophagy in HuH7 cells, and we have previously reported galangin-induced autophagy in HepG2 cells (Wen et al., 2012). In this study, we investigated whether galangin treatment activates TGF-β/Smad signalling to induce autophagy in HepG2 cells and determined a potential mechanism of activation.

2. Materials and methods

2.1. Cell culture

The human liver cancer cell line HepG2 was maintained at the Institute of Biochemistry and Molecular Biology at Guangdong Medical College. This cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% foetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere at 5% CO_2.

2.2. Agents and chemicals

Galangin (PubChem CID: 5281616) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) before being added to the cell cultures. The final concentration of DMSO in the culture medium was kept below 0.1% (v/v) after the addition of galangin. MTT and Hoechst 33258 were purchased from Sigma–Aldrich. LY2109761 was purchased from Selleck Chemicals (Houston, TX, USA). Rabbit, goat or mouse polyclonal antibodies against ATG16L, ATG12, ATG3, Beclin1, light chain 3(LC3), TGF-βRI, TGF-βRII, Smad1, Smad2, Smad3, Smad4, Smad6, Smad7, Actin and Beclin1 as well as siRNAs against ATG16L, ATG12, ATG3 and Smad4 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pSmad1, pSmad2 and pSmad3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.3. Cell viability assay

The cells were treated with galangin, and cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to a previous report (Wang et al., 2012).

2.4. Detection of autophagy by pEGFP-LC3

HepG2 cells were transfected with pEGFP-LC3, a highly specific fluorescence marker of autophagy, to measure autophagy levels. FuGENE HD (Roche Diagnostics, Basel, Switzerland) was used to transfect the HepG2 cells. After the induction of autophagy by galangin, the cellular localisation pattern of green fluorescence protein (GFP)-LC3 was photographed using a Nikon fluorescence microscope. When autophagy occurs, the percentage of GFP-LC3-positive cells with GFP-LC3 punctate foci increases, and the foci redistribute from a diffuse pattern to a punctate cytoplasmic pattern (GFP-LC3 foci) that specifically labels preautophagosomal and autophagosomal membranes, respectively.

2.5. Cell apoptosis and caspase-3 activity analysis

Apoptosis was evaluated by *in situ* uptake of Hoechst 33258 as reported by Zhang et al. (2012). The apoptotic index was determined by dividing the number of apoptotic nuclei by the total number of nuclei in each visual field ×100%.

In addition, the cells were collected after treatment by centrifugation, washed twice with PBS, and fixed with ice-cold 70% ethanol overnight. Prior to flow cytometry analysis for cell apoptosis, the fixed cells were washed once with PBS and incubated with 100 μg/ml propidium iodide plus 200 μg/ml RNase. The apoptotic level was determined by analysing the peak of the subG1 ratio.

Caspase-3 activity was measured using the Colorimetric Assay Kit according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). After treatment, the cells were collected and lysed in chilled lysis buffer, which contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM EDTA, 0.2% Triton X-100, 10 μg/ml aprotinin, 1 mM PMSF and 5 mM dithiothreitol, for 15 min on ice. The supernatant, containing 100 μg of protein, was incubated with 0.2 mM AcDEVD-pNA, a specific substrate for caspase-3. Caspase-3 activity was measured at 405 nm, with background subtraction at 570 nm using the Varioskan Flash Reader spectrophotometer (THERMO, MA, USA), and expressed as Ac-pNA cleavage or released absorbance.

2.6. Real-time polymerase chain reaction (RT-PCR)

The HepG2 cells were treated with 148 μM galangin for 24 h. Total RNA was isolated using Trizol reagent and digested with DNase I. A quantitative comparison of mRNA levels was conducted using real-time PCR with beta-actin as the endogenous control. The amplification reactions were carried out according to the one-step SYBR® prime Script® RT-PCR II kit instructions (perfect real-time

Table 1
Primer sequences for the genes.

Gene names	Primers
Beclin1	5′-GGTGTCTCTCGCAGATTCATC-3′5′-TCAGTCTTCGGCTGAGGTTCT-3′
ATG16L	5′-ACA TGATGGTGCGTGGAAT-3′5′-TTGTCCTTCTGCTGCATTTG-3′
ATG3	5′-GATGGCGGATGGGTAGATAC-3′5′-TCTTCACATAGTGCTGAGCAAT-3′
ATG12	5′-TAGAGCGAACACGAACCATC-3′5′-CACTGCCAAAACACTCATAGAG-3′
TGF-βRI	5′-GCCACAACCGCACTGTCA-3′5′-TGAACAAGCAATGGTAAACCTGAG-3′
TGF-βRII	5′-CTGTGGCCGCTGCACAT-3′5′-TTGTTGTCAGTGACTATCATGTCGTTA-3′
Smad1	5′-ACCTGCTTACCTGCCTCCTG-3′5′-CATAAGCAACCGCCTGAACA-3′
Smad2	5′-ACCGAAATGCCACGGTAGAA-3′ 5′-TGGGGCTCTGCACAAAGAT-3′
Smad3	5′-GCCTGTGCTGGAACATCATC-3′ 5′-TTGCCCTCATGTGTGCTCTT-3′
Smad4	5′-CATCCTGCTCCTGAGTATTGG-3 ′5′-GGGTCCACGTATCCATCAAC-3′
Smad6	5′-CTGCAACCCCTACCACTTCA-3′5′-TTGGTAGCCTCCGTTTCAGT-3′
Smad7	5′-AGAGGCTGTGTTGCTGTGAA-3′5′-AAATCCATCGGGTATCTGGA-3′
β-actin	5′-CCACACCTTCCTACAATGAGC-3′5′-TAGAGGAAGACGTAGGACAG-3′