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Oleanolic acid inhibits proliferation and invasiveness of Kras-transformed cells via autophagy

Jia Liu[a,c,1], Lanhong Zheng[b,1], Leina Ma[d,1], Bin Wang[e], Youguang Zhao[f], Ning Wu[c], Ge Liu[c], Xiukun Lin[c,g,*]

[a]College of Medicine, Qingdao University, Qingdao 266021, China
[b]Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China
[c]Institutes of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[d]Department of Molecular Biology, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China
[e]Department of Gastroenterology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing 400042, China
[f]Department of Urology, General Hospital of Chengdu Military Area Command of Chinese PLA, Chengdu 610083, China
[g]Capital Med. University, Dept. of Pharmacology, Beijing 100069, China

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Abstract

Oleanolic acid (OA) has been widely studied because of its pleiotropic therapeutic and preventive effect on various diseases. However, the mechanisms of OA's action are still not clear yet, especially its suppressing effect on transformed cells. In this work, we found that OA induced autophagy in normal tissue-derived cells without cytotoxicity. OA-induced autophagy was shown to decrease the proliferation of KRAS-transformed normal cells and to impair their invasion and anchorage-independent growth. Interrupting autophagy rescued OA's effect on the transformed cells. Mouse model experiments also demonstrated that OA suppressed the growth of KRAS-transformed breast epithelial cell MCF10A-derived tumor xenograft by inducing autophagy. Finally, we identified that OA induced autophagy in normal cells by inhibiting the activation of Akt/mTOR/S6K signaling. In conclusions, we found that OA treatment permitted normal cells to undergo autophagy. The induced autophagy was required for OA to prevent or delay the growth of transformed normal cells.

Keywords: Oleanolic acid; Autophagy; Akt; Cancer prevention; Natural compounds

1. Introduction

Oleanolic acid (OA) belongs to pentacyclic triterpenoid compounds and exists in various vegetables and medicinal herbs, in the form of aglycones or free acid. Its inhibitory activity on the progression, invasion, metastasis and angiogenesis of various cancer cells has been extensively studied [1]. Furthermore, OA was also shown to prevent carcinogenesis. Specifically, OA has been defined as one of the active components in *Prunella* extract that prevents the initiation of lung cancer [2]. Animal studies have demonstrated that OA is able to inhibit the promoting activity of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on skin cancer [3], and it has been applied to prevent this malignant disease in Japan [4]. In a rodent model, OA inhibits the preneoplastic lesion in colonic aberrant crypt foci, which usually progresses into colon cancer [5,6]. Synthetic OA derivatives have also been extensively shown to exert cancer-preventing activity [7–10]. However, the mechanism by which OA prevents or delays the growth of transformed cells is largely unknown.

* Corresponding author. Dept. of Pharmacology, Capital Medical University, Beijing 100069, China. Tel.: +86 10 83911837.
E-mail address: linxiukun@yahoo.com (X. Lin).
[1] These authors contributed equally to this work.

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Carcinogenesis involves one or more stable cellular changes caused by mutations in oncogenes, such as KRAS and CMYC, or exposure to carcinogens, such as diethylnitrosamine (DENA). These alterations, such as impaired cellular autophagy, predispose the affected cell and its progenies to subsequent malignant transformation [11].

Normal cells employ autophagy as means to survive the deprivation of nutrition and growth factors. Autophagy is also a mechanism by which cells degrade excessive and dysfunctional cellular components, such as proteins and organelles, via a lysosome-dependent fashion [12], thereby preventing the intracellular accumulation of cytotoxic molecules. Numerous evidences showed that basal autophagy plays a critical role in the maintenance of cellular homeostasis and genomic integrity [13].

A close association between interrupted autophagy and tumorigenesis has been uncovered in many studies in recent years. The mouse lacking the key autophagy promoter, Beclin-1, exhibits predisposition to tumorigenesis, suggesting that Beclin-1 contributes to the prevention of malignant transformation. Defect in autophagy was also been observed in spontaneous hepatocellular carcinoma (HCC) mouse model, in which mTOR complex 1 was constantly activated [14]. Consistently, tumor suppressor activity of autophagy was also proved in a transgenic mouse model that was susceptible to HCC [15]. In the TLR2-deficient mice, attenuated autophagy was

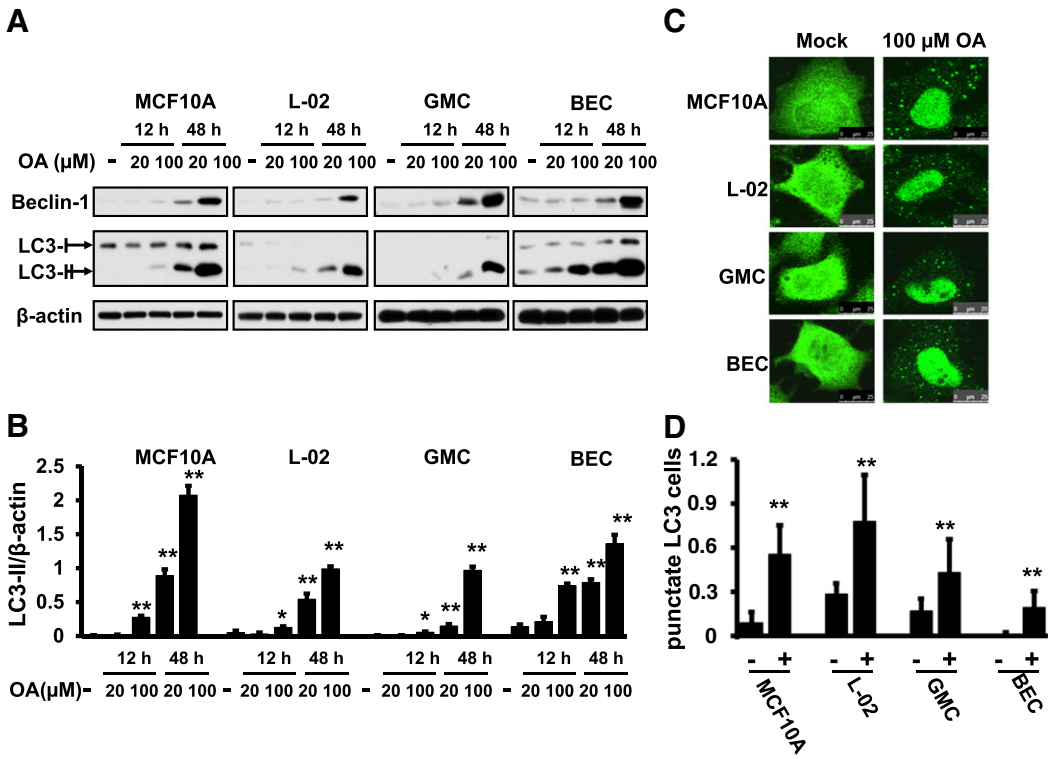

Fig. 1. OA induced autophagy in normal cells. (A) MCF10A, L-02, immortalized gastric mucosal and primary bladder epithelial cells were treated with 20 or 100 μM OA. After 12 or 48 h, the cells were harvested for detection of LC3 and Beclin-1 expressions with β-actin as endogenous reference. Immortalized gastric mucosal, GMC; Primary bladder epithelial cells, BEC. (B) The ratios of LC3-II to β-actin was calculated with ImageJ and expressed as mean±S.D. from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$. (C) MCF10A, L-02, immortalized gastric mucosal and primary bladder epithelial cells were transfected with pGFP-LC3 and treated with or without OA (100 μM). After 48 h, GFP-LC3 expression was observed by fluorescence microscopy. (D) The percentages of cells with punctate distribution of GFP-LC3 were quantified in GFP-positive cells from 10 random fields (expressed as mean±S.D. from three independent experiments). $^{**}P<0.01$.

observed along with the increased incidence of HCC initiation. Restoring autophagy was able to impair hepatic tumorigenecity induced by DENA [15]. Furthermore, more evidence has shown that autophagy suppressed the tumorigenesis in liver [16–18]. For instance, microtubule-associated protein 1 small form (MAP1S) has been identified to suppress the onset of hepatocellular carcinoma by enhancing autophagic event [17]. In addition to liver, other organs were also subjected to autophagy-induced protection against tumor initiation [19–21]. Ras-mediated malignant transformation of mouse NIH3T3 and embryo fibroblast cells was found to be promoted by defected autophagy [19]. Atg4C$^{-/-}$ mice showed an increased susceptibility to fibrosarcomas induced by carcinogen [20]. Akt kinase facilitated the formation of an autophagy-inhibitory Beclin-1/14-3-3/vimentin complex by phosphorylating Beclin-1, thereby increasing the malignant transformation [21].

In this study, we intended to identify the mechanism by which OA prevent or delay the growth of transformed normal cells, and to verify if autophagy is involved in OA-mediated cancer prevention.

2. Materials and methods

2.1. Cell lines and primary cell culture

Normal human breast epithelial cell line MCF10A was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM)/F-12 medium supplemented with 5% heat-inactivated horse serum (Invitrogen), 10 μg/ml insulin, 20 ng/ml EGF, 0.1 μg/ml cholera toxin, 0.5 μg/ml hydrocortisone, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Human normal liver cell line, L-02, was purchased from Shanghai Cell Collection (Shanghai, China) and cultured using DMEM supplemented with 10% of fetal bovine serum (FBS), 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml.

To establish the primary culture of gastric mucosal cells (GMC), fresh tissue was obtained from upper G.I. biopsies of informed patients according to protocols approved by Ethical Review Board in the Third Military Medical University (Chongqing, China). Gastric mucosa were processed following the previous procedures [22]. The primary cultures were established initially in RPMI-1640 media supplemented with 20% FBS and maintained in RPMI-1640 media supplemented with 10% FBS. The cells were then immortalized with adenoviral vector, Adeno-SV40 (Applied Biological Materials, Canada), according to the manufacturer's instructions.

For human primary bladder epithelial cell culture, the samples were obtained from a patient that underwent cystoscopic examination of asymptomatic hematuria (the biopsies were not malignant revealed by histopathological results). The previously described procedure was followed [23], which has been approved by ethical review board in General Hospital of Chengdu Military Area Command of Chinese PLA (Chengdu, China). The patient approved the application of their samples for this study.

All the cells were cultured in a humidified 5% CO_2-containing atmosphere at 37°C.

2.2. Chemical reagents

OA was purchased from Sigma-Aldrich (analytical standard). Autophagy inhibitor 3-methyladenine (M9281), mTOR inhibitor rapamycin (R0395) and mTOR stimulator IGF-1 (I3769) were also purchased from Sigma-Aldrich.

2.3. pGFP-LC3 transfection

The cells were transfected with the plasmid, pEGFP-LC3, using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instruction, followed by being cultured on coverslips in a 24-well plate. After transfection for 48 h, cells were treated with 100 μM OA for another 48 h. Cells were fixed with 4% Polyoxymethylene for 20 min, and then, were observed under Olympus FV1000 confocal microscope (Olympus, Germany). The excitation wave length is 475 nm and the emission wave length is 509 nm. The cells that contained more than five GFP-LC3 puncta were counted in 10 randomly selected fields.