Full Paper

Augmentation of Oridonin-Induced Apoptosis Observed With Reduced Autophagy

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Abstract. Our previous studies showed that oridonin could induce apoptosis in HeLa cells; and in this study, we further investigated autophagy induced by oridonin in HeLa cells and the relationship between apoptosis and autophagy. HeLa cells were exposed with oridonin after 3-methyladenine (3-MA) pre-culture, and we evaluated the growth inhibitory ratio, morphologic changes, DNA fragmentation, proteins expression as well as autophagic and apoptotic levels. Oridonin inhibited the proliferation of HeLa cells in vitro and induced autophagy. Oligonucleosomal fragmentation of DNA as well as increased activities of Bax proteins were induced by oridonin, but the expression of p-Bcl-2 protein was reduced. In the condition of oridonin-treatment, when the inhibitor of phosphoinositide 3-kinase (PI3K), wortmannin, was applied, the autophagic level was significantly decreased, while the apoptotic level was increased, indicating that PI3K is a key regulator of both autophagy and apoptosis. Akt, down-stream factor of PI3K, was activated in autophagic process but suppressed in apoptosis in this study. In addition, when autophagy was blocked by 3-MA, the expression of SIRT-1 was decreased, indicating SIRT-1 contributed to autophagy. Taken together, oridonin simultaneously induced HeLa cell both apoptosis and autophagy in HeLa cells, and inhibition of autophagy contributes to upregulation of apoptosis.

Keywords: oridonin, apoptosis, autophagy

Introduction

Oridonin was purified from *Rabdosia. Rubescens*, a member of the salvia family, and oridonin (Fig. 1A) has been traditionally used for treatment of various diseases such as leukemia in China (1 – 3). It has various biological, pharmaceutical, and physiological functions such as anti-cancer activity, anti-bacteria activity, and disinsection (4, 5). Oridonin has inhibitory effects on the synthesis of both deoxyribonucleic acid and anti-apoptotic proteins, such as Bcl-2 (6), and its Kaurene structure is thought to be the active domain for anti-cancer function (7, 8).

Programmed cell death is used to selectively remove unwanted or dangerous cells in multicellular animals.

On the basis of the morphology, two major forms can be recognized: apoptosis- and autophagy-type cell death. Apoptosis serves as a targeted elimination of individual cells during physiological circumstances, followed by endocytosis of cellular remnants by macrophages and subsequent degradation in lysosomes (9, 10). Autophagy is a conserved vacuolar trafficking pathway in eukaryotes that targets subcellular constituents to the vacuole for degradation and recycling. It is a bulk cytoplasm degradation process, resulting in the digestion of self-material by lysosomal hydrolases. The main and best-known step of autophagy is an isolation membrane of endoplasmic reticulum origin that engulfs portions of the cytoplasm, including organelles (11 – 13). The emerging autophagosome fuses with a lysosome, forming an autophagic vacuole, in which the sequestered material is degraded (14 – 16). 3-Methyladenine (3-MA) is an agent long known to selectively and potently inhibit

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Fig. 1. Chemical structure (A) and HPLC profile (B) of oridonin. Quantitation and determination of oridonin were performed by HPLC (HITACHI L-7400; Hitachi, Tokyo) using a column (Diamonsil C18 250 × 4.6 mm, 5 μm; Dikma Technologies, Beijing, China) at 30°C with MeOH-H₂O (65:35) as the mobile phase at 1 mL/min and detection of the elute at 238 nm. The retention time (RT) for oridonin was 7.173 min.

autophagy-dependent protein degradation and suppress the formation of autophagosomes. Both apoptosis and autophagy are vital biological processes in cells, and apoptosis has been studied for several decades, while autophagy is a new focus for recent studies and achieved satisfying progress. However, research about the relationship between apoptosis and autophagy is just taking a step forward.

The Bcl-2 family, including apoptosis promoting proteins Bax and Bid, as well as apoptosis suppressing proteins Bcl-2 and Bcl-XL, has an important function in controlling apoptosis. The balance between these two groups settles the fate of cells in many programmed cell death systems (17, 18). The Bax gene is a member of the family that enhances apoptosis to limit cell proliferation, and it can be negatively regulated during progression of many human cancers, suggesting that it may act as a tumor suppressor, that is dependent on p53 activation (19). Bcl-2 is an anti-apoptotic protein that prevents the activation of caspase-3 (20), and it controls the size of the mitochondrial permeability pore in order

to regulate the release of apoptosis-related factors such as cytochrome *c* (21, 22). SIRT1 is histone deacetylase and is a vital regulators of fundamental cellular events, including cell cycle progression, differentiation, and tumorigenesis (23, 24). Bax tumor suppressor gene is downregulated by SIRT1 through the DNA end binding factor Ku70. In the absence of SIRT1, Ku70 might be hyper-acetylated, leading to disruption of Ku70-Bax interaction. This in turn releases more Bax, which promotes apoptosis (25).

The phosphoinositide 3-kinase (PI3K) enzymes, known as anti-apoptosis kinases, regulate several signal transduction pathways controlling vital cell process that are implicated in carcinogenesis. Initial studies implicated both PI3K and Akt in the prevention of apoptosis (26). Signaling through PI3K and the downstream kinase Akt has been widely implicated as an important survival signal in cell proliferation (27–30). 3-MA was recently classified as a class III PI3K inhibitor (31), which indicates there are some links between PI3K/Akt and autophagy.

Materials and Methods

Chemical reagents

Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The purity of oridonin isolated from *Rabdosia rubescens* was confirmed by HPLC to be higher than 99% as shown in Fig. 1B. Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The DMSO concentration was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death.

Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); monodansylcadaverine (MDC), 3-methyladenine (3-MA), wortmannin, propidium iodide (PI), and RNase A were purchased from Sigma Chemical (St. Louis, MO, USA); thiazolyl blue (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was from Sino-American Biotechnology (Beijing, China); rabbit polyclonal antibodies against Bax, Akt, and phospho-Akt (p-Akt), mouse polyclonal antibodies against Bcl-2 and phospho-Bcl-2, and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit or goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The cervical carcinoma HeLa cell line was obtained from American Type Culture Collection (#CRL 1872; ATCC, Rockville, MD, USA). The cells were cultured

in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS and 0.03% L-glutamine (Hyclone, Logan, UT, USA) and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Cell growth assay

The cytotoxic effect of oridonin on HeLa cells was measured by MTT assay as described previously (32). The cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^5 cells per well. After 24 h incubation, they were treated with various concentrations of oridonin for different periods of time. Four hours before the end of incubation, 20 μ L MTT solution (5.0 mg/L) was added to each well. The resulting crystals were dissolved in DMSO. Optical density was measured by MTT assay using a plate microreader (TECAN SPECTRA, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell death (\%)} = \{1 - [A492 (\text{control}) - A492 (\text{oridonin})] / A492 (\text{control})\} \times 100$$

Observation of morphologic changes

HeLa cells (3×10^5) in RPMI 1640 containing 10% FBS were seeded into 6-well culture plates and cultured for 24 h. 3-MA at 2 mmol/L was added 1 h before 64 μ mol/L oridonin treatment. The cellular morphologic changes were observed using phase contrast microscopy (Leica, Nussloch, Germany) at 24 h.

Effect of wortmannin on autophagy and growth inhibitory rate

HeLa cells (1×10^6) were added with 200 nmol/L wortmannin (the inhibitor of PI3K) 1 h before 64 μ mol/L oridonin treatment. After 24 h, the cells were harvested. For measuring autophagy, the cell pellet was suspended with 0.05 mmol/L MDC at 37°C for 60 min, and then the samples were analyzed by flow cytometry analysis. For measuring the growth inhibition, the collected cells were examined by the MTT method.

Flow cytometric analysis of autophagy and cell cycle distribution

HeLa cells were treated with 2 mmol/L 3-MA 1 h before 64 μ mol/L oridonin treatment. After 24 h, cells were harvested by trypsin and rinsed with PBS by centrifugation at $1000 \times g$. Here we tested both autophagic ratio and cell cycle distribution. In brief, for measuring autophagy, the resulting cell pellet from 1×10^6 cells was suspended with 0.05 mmol/L MDC at 37°C for 60 min as described previously (33). On the other hand, for measuring apoptosis, the collected cells from 2×10^6 cells were fixed with 500 μ L PBS and

10 mL 70% ethanol at 4°C overnight; then after washing twice with PBS, the cells were stained with 1.0 mL PI solution containing PI 50 mg/L and RNase A 1 g/L.

These two samples were both analyzed by flow cytometry (FACScan; Becton Dickinson, Sunnyvale, CA, USA) using the Cell Quest software (Becton Dickinson) to determine the percentage of cells undergoing autophagy and of cells at different phases of the cell cycle.

DNA extraction and detection of DNA fragment

DNA extraction and electrophoresis were performed as described previously (34). In brief, 2×10^6 HeLa cells containing adherent and floating ones were collected by centrifugation at $1000 \times g$ for 5 min. The cell pellet was suspended in cell lysis buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L edetic acid pH 8.0, 0.5% Triton-X 100) and kept at 4°C for 30 min. The lysate was centrifuged at $25,000 \times g$ for 20 min. The supernatant was incubated with RNase A (40 μ g/L) at 37°C for 1 h and then incubated with proteinase K (40 μ g/L) at 37°C for 1 h. The resulting solution was mixed with 5 mol/L NaCl and dimethylcarbinol overnight at -20°C and then centrifuged at $25,000 \times g$ for 15 min. After drying, DNA was dissolved in TE buffer (10 mmol/L Tris-HCl, pH 7.5) and separated by 2% agarose gel electrophoresis at 100 V and ethidium bromide (EB) staining.

Western blot analysis

HeLa cells (2×10^6) were preincubated with or without 2 mmol/L 3-MA for 1 h and then treated with 64 μ mol/L oridonin for 2, 6, 12, 24, or 48 h. Both adherent and floating cells were collected and Western blot was performed as previously described (35). Briefly, HeLa cells were washed with ice-cold PBS and solubilized with lysis buffer (1% SDS, 1 mmol/L phenylmethyl-sulfonylfluoride (PMSF), 1 mmol/L EDTA, 2 mmol/L leupeptin, and 1 mmol/L aprotinin). Protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein lysates were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were soaked in blocking buffer (5% skimmed milk), and then incubated with primary antibodies, followed by horseradish-peroxidase-conjugated secondary antibodies overnight. The color was developed with diaminobenzidine (DAB).

Statistical analysis

All data and results presented were confirmed in at least three independent experiments. The data are expressed as means $\pm$ S.D. Statistical comparisons were made by Student's *t*-test. $P < 0.05$ was considered significant.

Results

Cytotoxicity of oridonin on HeLa cells

Oridonin induced HeLa cell death in a time- and concentration-dependent manner. Oridonin from 20 to 160 $\mu\text{mol/L}$ exerted a potent inhibitory effect on HeLa cell growth. By 24 h after treatment with 64 $\mu\text{mol/L}$ oridonin, cell death rate reached to almost 50%. The IC_{50} for 24 h oridonin treatment was 63.2 $\mu\text{mol/L}$. Therefore, 24 h incubation with oridonin seemed to be sufficient for half induction of cell death. When HeLa cells were treated with oridonin at 160 $\mu\text{mol/L}$ for 8 h, about 50% of the cells died; for 48 h, the inhibitory ratio almost reached 100% (Fig. 2).

Oridonin induces apoptosis in HeLa cells

When tumor cells were treated with 64 $\mu\text{mol/L}$ oridonin for 24 h with or without 3-MA pre-culture, remarkable morphological changes were observed compared with the control group and 3-MA group (Fig. 3). By 24 h, the majority of HeLa cells had become round with shrunken nuclei. Some of these cells showed

membrane blebbing and nuclei were fragmented into apoptotic bodies (Fig. 3: B and C). The cells in control group and 3-MA treated group did not show these typical apoptotic features (Fig. 3: A and D).

Fig. 2. Time and dose curve of cell death by oridonin treatment. HeLa cells were treated with oridonin at various doses for 12, 24, 36, and 48 h. $n = 6$. Means $\pm$ S.D.

Fig. 3. Oridonin induced morphologic changes of HeLa cells. Cellular morphology were examined in the absence of oridonin (A) ($\times 200$ magnification), in the presence of oridonin (B) ($\times 200$ magnification), in the presence of oridonin and 3-MA (C) ($\times 200$ magnification), or in the presence of 3-MA (D) ($\times 200$ magnification); and E, F $\times 400$ magnification. Arrow: the apoptotic HeLa cell displaying apoptotic bodies and membrane blebbing. Bar = 20 μm .

PI3K facilitates autophagy and suppressed apoptosis

When HeLa cells were pre-treated with 200 nmol/L wortmannin for 1 h, followed by 64 μ mol/L oridonin treatment, the autophagic ratio 24 h after oridonin introduction was significantly reduced compared with the oridonin alone treated group (Fig. 4A), whereas inhibitory ratio of cell proliferation was significantly increased (Fig. 4B). When PI3K activity was reduced, apoptotic ratio was augmented, on the other hand, the autophagic ratio was decreased, indicating that PI3K antagonized apoptosis, but promoted autophagy.

Inhibition of autophagy facilitates upregulation of apoptosis induced by oridonin

Oridonin at 64 μ mol/L could induce both autophagy and apoptosis simultaneously. 3-MA, a specific inhibitor of autophagy, potently suppressed oridonin-induced autophagy (Fig. 5A). When autophagy was suppressed by 3-MA in oridonin-treated HeLa cells, cell death ratio and apoptosis were significantly enhanced compared with the oridonin alone treatment group. Compared with the oridonin alone treatment group, the number of apoptotic cells was larger in the 3-MA and oridonin treatment group, and the apoptotic features, including apoptotic bodies, membrane blebbing, cell becoming round and floating, were more significant (Fig. 5: B and C), suggesting that autophagy antagonized apoptosis induced by oridonin in HeLa cells.

Fig. 4. The effects of wortmannin on autophagy and apoptosis. A: Autophagy was suppressed by wortmannin application. B: Apoptosis was promoted when wortmannin was introduced.

Oridonin induced DNA fragmentation in HeLa cells

Internucleosomal DNA fragmentation was observed in HeLa cells induced by 64 μ mol/L oridonin by agarose gel electrophoresis. DNA fragmentation was significantly augmented by combined treatment with 2 mmol/L 3-MA (Fig. 6).

3-MA employment reinforced the enhancement of the Bax/Bcl-2 balance and the downregulation of SIRT1 induced by oridonin

To assess whether autophagy participates in the apoptotic pathway, the specific autophagic inhibitor 3-MA was introduced. HeLa cells were pretreated with 2 mmol/L 3-MA for 60 min and cultured with 64 μ mol/L oridonin for fixed times, then Bax, Bcl-2, and phospho-Bcl-2, expression were detected. Bax expression was increased time-dependently by oridonin treatment (Fig. 7A), while Bcl-2 phosphorylation and SIRT1 expression were suppressed (Fig. 7: B and D), compared with the control and 3-MA treated group in which expression of those proteins were unaffected; notably, these effects were remarkably reinforced when 3-MA applied. Furthermore, Bcl-2 was stably expressed in all of the four groups (Fig. 7C).

The expression of p-Akt was suppressed in apoptosis but enhanced in autophagy

HeLa cells were cultured with 64 μ mol/L oridonin for fixed times and with 2 mmol/L 3-MA preculture for 60 min, and then Akt and p-Akt expression were detected. Oridonin and 3-MA treatments had no effects on the genetic expression of Akt (Fig. 8A). p-Akt expression was decreased time-dependently with oridonin treatment and this suppressive effect was more significant when autophagy was blocked by 3-MA pre-treatment.

Discussion

Apoptosis is regulated by a complex network of molecules whose expression could have a marked influence on chemosensitivity. Bcl-2 is a 26 kDa protein located in the inner mitochondrial membrane (36, 37), and it is a member of a family of homologous proteins that uniquely regulates apoptosis (38, 39). Bax can bind Bcl-2 and the consequent formation of heterodimer provides protection against apoptosis. Hence, Bcl-2/Bax ratio is important in apoptosis induced by several agents and the decline of this ratio contributes to apoptosis induction. In addition, Bax/Bax interactions of Bax dimers or oligomers directly form channels in mitochondrial outer membrane in apoptotic development (40).

Fig. 5. The relationship between apoptosis and autophagy induced by 64 $\mu\text{mol/L}$ oridonin. A: Autophagic ratio induced by oridonin and suppressed by 3-MA. B: The inhibitory effects of oridonin and 3-MA on HeLa cell growth. $*P < 0.05$. C: Inhibition of autophagy enhanced apoptotic level. Top: histograms of flowcytometer for cell cycle distribution of HeLa cells. Bottom: the corresponding linear diagram of the upper histograms.

Fig. 6. DNA fragmentation induced in HeLa cells. M: marker, lane 1: medium control, lane 2: oridonin and 3-MA treatment, lane 3: oridonin treatment, lane 4: 3-MA treatment.

It was previously suggested that autophagy and apoptosis were distinct forms of cell death, but more recent data seems to imply that there is a mechanistic overlap between the two archetypes that represent a continuous spectrum of different shades of physiological cell death (41). Autophagy has been postulated to be a primary cell response to apoptotic inducers and when the autophagic capacities are overloaded, mitochondrial pro-apoptotic factors would activate the death program (42). Some observations indicate that autophagy has functions in helping cells to escape apoptosis through the sequestration of cytochrome *c* (43). Other evidences showed that active autophagy appeared to increase the tendency to undergo apoptosis, and both correlate with the mitochondrial permeability transition in certain

Fig. 7. The expression of Bax, Bcl-2, and SIRT1 in oridonin treated HeLa cells. The cells were treated with 64 $\mu\text{mol/L}$ oridonin for 2, 6, 12, 24, and 48 h. A: Bax, B: phospho-Bcl-2, C: Bcl-2, D: SIRT1.

Fig. 8. The expression of Akt in oridonin treated HeLa cells. The cells were treated with 64 $\mu\text{mol/L}$ oridonin for 2, 6, 12, 24, and 48 h. A: Akt, B: p-Akt.

cells. This is in agreement with other cases where autophagy is required for efficient apoptosis. For example, Peng and his colleagues reported inhibition of autophagy decreased the apoptosis induced by vincristine (44–46). Xue and his colleagues also reported that in newly isolated sympathetic neurons, two independent apoptotic stimuli, NGF-deprivation or cytosine arabinoside added in the presence of NGF, caused remarkable augmentation of autophagy, and the anti-autophagic drug 3-methyladenine also delayed apoptosis (47). In this study, we first showed that autophagy could antagonize apoptosis in the condition of oridonin treatment. The function of autophagy and its links to apoptosis are disputed problems. Concludingly, plenty of signaling pathways overlaps are found between autophagy and apoptosis, including various kinases such as PI3K, PKB/Akt, Bcl-2 family members, PTEN, c-Myc, Ras and others (48–52).

Here, our results from growth inhibition measurement, flow cytometric analysis, DNA fragmentation assay, and Western blotting showed that employing 3-MA, the specific inhibitor of autophagy pathway, enhanced the apoptotic level, which differed from previous reports and this phenomenon might be specific to oridonin.

The apoptotic protein Bax, was activated by oridonin treatment, while anti-apoptotic Bcl-2 activity and SIRT1 expression were suppressed, and these effects were remarkably reinforced when autophagy was inhibited by 3-MA. Notably, the expression of p-Bcl-2 in the oridonin and 3-MA treatment group was significantly augmented rather than reduced, compared with the oridonin alone treatment group, suggesting that p-Bcl-2 participated in the autophagy process and acted as a suppressive factor of autophagy here. We thought that it was the balance of Bax and Bcl-2 that controlled apoptosis, whereas phospho-Bcl-2 regulated autophagy. Moreover, when autophagy was blocked by 3-MA, the expression of SIRT1 was decreased while Bax expression was increased, indicating that both SIRT1 and Bax participated in autophagy, that is, SIRT1 contributed to autophagy, whereas Bax inhibited autophagy.

When the inhibitor of PI3K, wortmannin, was applied, the autophagic level was significantly decreased, while the apoptotic level was increased, indicating that PI3K is a key regulator of both autophagy and apoptosis, which functions as a fork point of these two signaling pathways.

The serine/threonine kinase, Akt (protein kinase B), is a downstream effector of PI3K. The activation of this pathway allows cells to survive by the inhibition of proapoptotic signals and the induction of survival signals, which may contribute to malignant transforma-

tion and tumor aggressiveness (53). Akt was activated in an autophagic process, but suppressed in apoptosis in this study. Thus, we can conclude that the PI3K/Akt pathway is activated in autophagy, but inhibited in apoptosis.

From these evidences, we could draw a conclusion that there are many crosstalks between autophagy and apoptosis in oridonin-treated HeLa cells. Many proteins, including Bax, Bcl-2, PI3K, Akt, and SIRT1, were related to both apoptosis and autophagy. However, the specific associations among these proteins are still not clear. In summary, PI3K/Akt signaling is augmented in autophagy, while it is suppressed in apoptosis. SIRT1 controls the reaction of Bax/Ku70, resulting in altering the balance of Bax/Bcl-2, which regulates the fate of cells, that is, undergoing apoptosis or not. In addition, the anti-apoptotic Bcl-2 activity was inhibited in the autophagic pathway. Consequently, autophagy could protect HeLa cells from death through antagonizing apoptosis induced by oridonin. Autophagy was a protective mechanism for HeLa cells stimulated by oridonin.

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