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Original Article

Mechanisms of Raddeanin A-induced autophagy and apoptosis in human colorectal cancer cells

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Abstract: Several studies have confirmed that the Chinese medicine Raddeanin A (RA), which is extracted from the plant Anemone raddeana Regel, can inhibit the proliferation of a variety of tumor cell lines. Previously, our team demonstrated that RA could induce apoptosis and autophagy in human gastric cancer cells. Objective: This experiment was intended to explore whether RA can induce autophagy and apoptosis in human colorectal cancer HCT116 cells and to investigate the mechanisms and relationship between autophagy and apoptosis. Methods: Cell proliferation was detected via MTT assay. Transmission electron microscope was used to observe autophagosomes as a marker for autophagy. Apoptosis was examined via Hoechst 33258 staining. Flowcytometry was used to calculate the rate of apoptosis. The expression of related genes and proteins were tested by reverse transcription-polymerase chain reaction (RT-PCR) and western blot, respectively. Results: Cell viability gradually decreased with an increase of drug concentration and disposal time. This result indicated that RA could significantly inhibit the growth of HCT116 cells in a manner that was both time-and concentration dependent. RA induced autophagy and apoptosis in HCT116 cells. RT-PCR and western blot showed that the expression of genes and proteins related to autophagy increased. Moreover, the expression of proteins that suppress apoptosis decreased while pro-apoptotic protein levels increased. The expression of proteins involved in the PI3K-AKT-mTOR signaling pathway also decreased, while caspase-8 and 9 levels increased. Compared with RA treatment alone, the apoptosis rate decreased when HCT116 cells were treated with RA and hydroxychloroquine (HCQ) together. However, when RA and rapamycin (RAPA) were given together, the apoptosis rate increased. Conclusion: RA induces autophagy by regulating the PI3K-AKT-mTOR signaling pathway and apoptosis through intrinsic and extrinsic pathways. Autophagy induced by RA in HCT116 cells can promote cell apoptosis.

Keywords: Raddeanin A, autophagy, apoptosis, HCT116 cells, induce

Introduction

Colorectal cancer is a common malignant tumor of the digestive system. In Europe and the United States, the morbidity and mortality of colorectal cancer are in fourth and second place, respectively, in all malignant tumors [1]. For the treatment of colorectal cancer, chemotherapy is commonly used and is effective treatment after surgery. Unfortunately, chemotherapy can cause problematic adverse reactions, and drug resistance can occur in cancer cells, which greatly reduces the clinical effect [2]. Consequently, for new natural antineoplastic drugs are needed.

Raddeanin A (RA) is an oleanane-type triterpenoid saponin extracted from the plant *Anemone raddeana* Regel, which was recorded in *YIFANGGEKUO* as an effective treatment for breast cancer. Modern pharmacological studies have shown that it has significant antitumor, anti-inflammatory, antipyretic, analgesic, and anticonvulsant effects [3-6]. Moreover, our previous studies demonstrated that RA inhibited the invasion of SGC-7901 human gastric cancer cells into healthy tissue in vitro, and induced autophagy and apoptosis in those cancer cells [7, 8].

Autophagy, orprogrammed cell death II (PCDII), is a separate process from apoptosis (PCDI),

Table 1. Sequences of primers used in the reverse transcription-polymerase chain reaction (RT-PCR) amplifications

Gene primer	Sequence (5'-3')	Length of PCR product (bp)
Beclin-1	F: GACGGAAGTTGAGATAGT	110
	R: CAAGTGACGAAACGGTGATT	
ATG 5	F: GATGAGGGCCGTATCGACAGT	160
	R: CGCTTCGCTAAATTAGGCGAC	
ATG12	F: GCCATCGCGAAGTGCAAGAC	158
	R: ACCAGAAATATACACAGGGTCT	
ATG7	F: CAGCCTGCATTTAAGACCAGTGTCAC	210
	R: ACGTCGATCGCTCACACATGCATTCGCATT	
β-actin	F: GGCCAACCGCGAGAAGAT	134
	R: CGTCACCGGAGTCCATCA	

and also allows cells to die in an orderly fashion. PCDII has become a hot research topic recently with the hope of learning information to regulate tumor cell death. This experiment explored whether RA could induce autophagy or apoptosis in colorectal cancer HCT116 cells, investigated the possible molecular mechanisms of these processes, and attempted to determine the relationship between autophagy and apoptosis as induced by RA.

Materials and methods

Reagents and antibodies

RPMI-1640 medium and fetal bovine serum (FBS) were acquired from Gibco BRL (Gaithersburg, MD, USA). RA was purchased from the China National Institute for the Control of Pharmaceuticals and dissolved in dimethyl sulfoxide (DMSO) which was stored at -20°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and hydroxychloroquine sulfate were obtained from Sigma Chemical Company (St. Louis, MO, USA). Annexin-V/propidium iodide (PI) apoptosis detection kits were obtained from BD Biosciences (FranklinLakes, NJ, USA). Primescript reverse transcription reagent kits with gDNA erasers were obtained-from TaKaRa (Dalian, China). TRIzol reagent and Power SYBR Green PCR Master Mixes were bought from Life Technologies (Grand Island, NY). Rapamycin (RAPA) and primary antibodies-of Beclin-1, LC3, BAX, Bcl-2, PARP, caspase-3, cleaved-caspase-3, caspase-9, caspase-8, PI3K, AKT, p-AKT, mTOR, p-mTOR, andβ-actin were obtained from Cell Signaling Technology (Beverly, MA, USA), Fluorescein-conjugated secondary antibodies were obtained from Odyssey (Licor, Belfast, ME, USA).

Cell line and culture

The human colorectal cancer cell line (HCT116) was provided by the Shanghai Institute for Biological Research (Shanghai, China) and cultured in RPMI-1640 medium containing 10% FBS at 37°C and in a humidified atmosphere with 5% CO_2.

MTT assay

Cells in the logarithmic growth phase were plated in 96-well culture plates. Afterbeing treated with different concentrations of RA or DMSO, cells were added to MTT (5 mg/mL) and incubated for 4 hr. DMSO was then added and the optical densities (ODs) were tested using an ELx800 microplate reader (BioTek, Winooski, VT, USA) at 490 nm. The inhibition rate was determined according to the following equation: Inhibition rate = (1-$OD_{experiment}$/$OD_{control}$) * 100%.

Transmission electron microscopy (TEM) analysis

Cells were seeded in a Petri dish, incubated with RA for 12 h, and collected. The cells were then washed twice with cold PBS and fixed in 2. 5% glutaric dialdehyde solution and 1% osmic acid for 2 h. Afterthis, the processed cells were observed under a JEOL-1010 electron microscope.

Flow cytometry analysis

The cells were detached via trypsinization, treated with RA for 12 h, washed twice with PBS, and then resuspended in 500 μL binding buffer containing 5 μL Annexin V-FITC and 5 μL propidium iodide (PI). Prior to being analyzed by flow cytometry, the cells were incubated for 15 min in the dark.

Hoechst 33258 staining

After being treated with RA for 12 h, cells were fixed with 1% formalin for 30 min, washed with PBS, and incubated in Hoechst 33258 stain (50 ng/mL) for 30 min. Cells in the process of apoptosis could be distinguished by condensa-

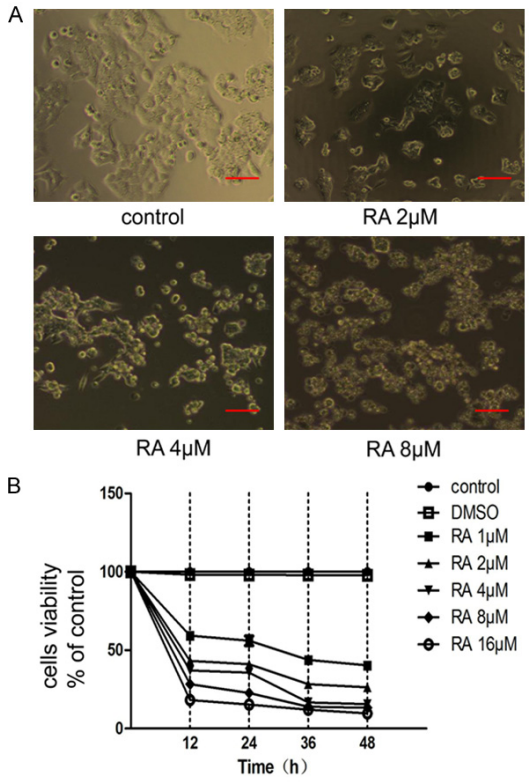

Figure 1. Raddeanin A (RA) inhibits HCT116 cell proliferation. A. Treatment with different concentrations of RA (2, 4, 8 μM) changed the morphology of the cells (200×). B. An MTT assay showed that cellular viability was positively correlated with RA concentration (1, 2, 4, 8, or 16 μM) and incubation time (12, 24, 36, or 48 h). Bar = 50 μm.

tion and fragmentation in their nuclei. The cells were recorded using a Zeiss Axioplan 2 fluorescent microscope (Jena, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Cells of different groups were collected, and treated with TRIzol reagent according to the manufacturer's instruction in order to extract the cellular RNA. The purity of the RNA was tested via spectrophotometer and reverse-transcribed into cDNA using a TaKaRa RT retrovirus kit. The date was calculated with an ABI 7500 fast RT-PCR System after the PCR amplification reaction. The $2^{-\Delta\Delta Ct}$ method was used to analyze the results, according to the following equations:

$$\Delta Ct = Ct_{\text{target genes}} - Ct_{\text{endogenous reference gene}} \quad (1)$$

$$\text{and } \Delta\Delta Ct = \Delta Ct_{\text{treated samples}} - \Delta Ct_{\text{control samples}} \quad (2)$$

where β-actin was used as the reference compound. The final gene expression level was calculated as $2^{-\Delta\Delta Ct}$. The gene primers were designed by Primer Express and are shown in **Table 1**.

Western blot (WB) analysis

After being treated with RA, the cells were lysed with RIPA buffer to release the protein. The protein concentration was tested via the Bradford method (BCA). The expression of β-actin protein was served as a loading control, andeach group proteins was loaded onto a 10% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis. Thereafter, the proteins were transferred via electroblotting to polyvinylidenedifluoride (PVDF) membranes (Millipore, Boston, MA, USA). The PVDF was enclosed with 5% BSA for 1 hr and incubated with the indicated antibodies against LC3 (1:1000), Beclin-1 (1:1000), BAX (1:1000), Bcl-2 (1:1000), caspase-3 (1:1000), cleaved-caspase-3 (1:1000), caspase-8 (1:1000), caspase-9 (1:1000), PARP (1:1000), PI3K (1:1000), AKT (1:1000), p-AKT (1:1000), mTOR (1:1000), and p-mTOR (1:1000) overnight. Before the secondary fluorescent antibody (1:3000 dilutions) was added for 1 h, the membrane was washed thrice (5 min/time) with tris buffered saline with Tween-20 (TBST). Finally, the signal intensity of the membranes was examined by Odyssey (LICOR, Belfast, ME, USA).

Statistical analysis

SPSS16.0 statistical software was used to perform the statistical analysis. Measurement data were represented as means ± standard deviation (SD). The difference between the groups was examined by one-way ANOVA analysis followed by Dunnett's test and a P value <0.05 was considered significant.

Results

RA treatment inhibited HCT116 cell proliferation

The MTT assay indicated that RA could significantly inhibit the proliferation of HCT116 cells. The effect on cellular proliferation different with concentration of RA (1, 2, 4, 8, 16 μM) and length of incubation (12 h, 24 h, 36 h, 48 h). DMSO had the smallest effect on cells viability (**Figure 1B**). Observations of HCT116 cells

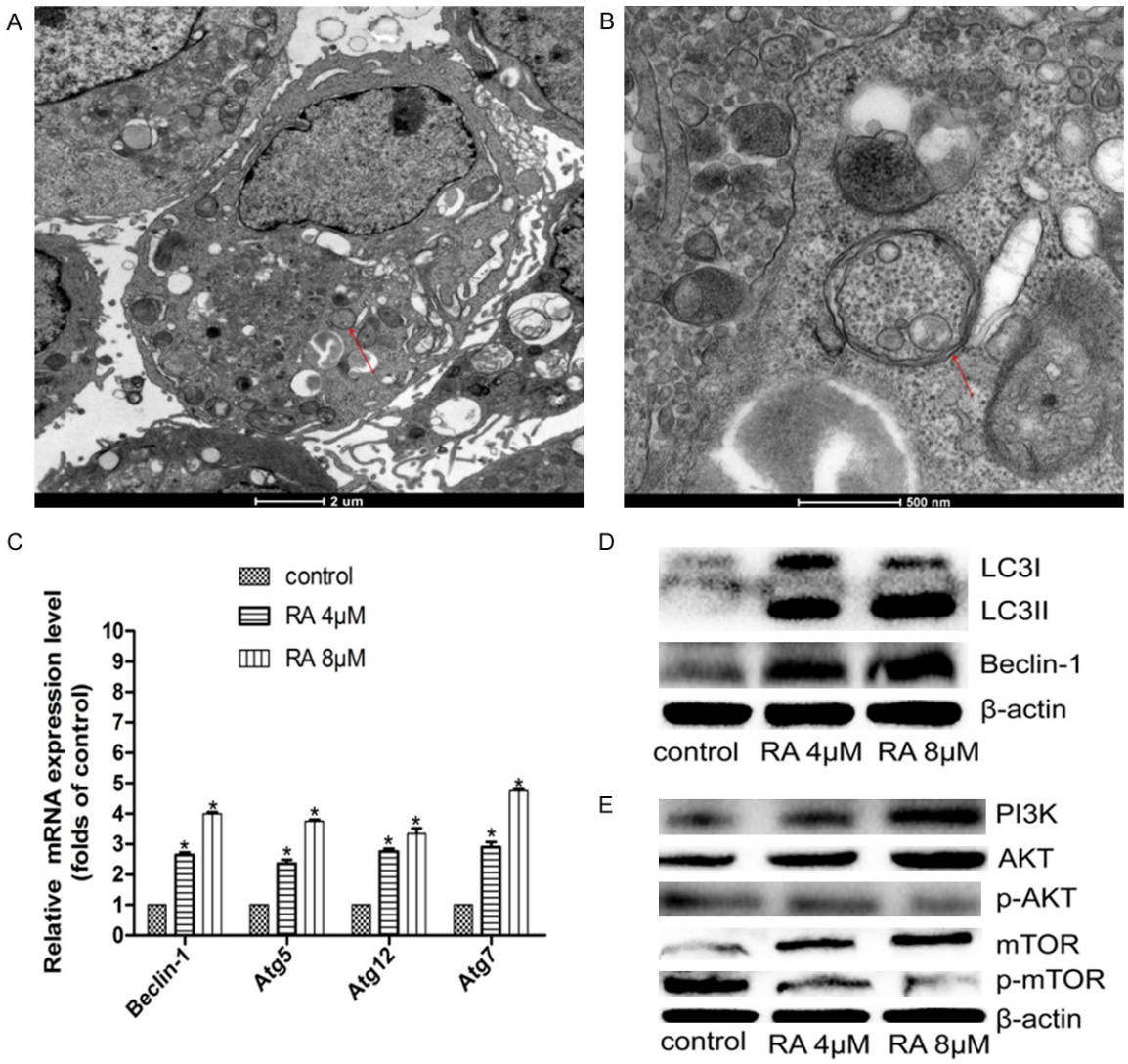

Figure 2. Raddeanin A (RA) induced autophagy in HCT116 cells by activating the PI3K-AKT-mTOR pathway. A. Cells were treated with RA (4 μM) for 12 h and transmission electron microscopy (TEM) was used to observe autophagosomes (2900×). B. A double membrane structure was detected using TEM (18500×). C and D. After being treated with RA (4, 8 μM), reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis showed the levels of autophagy related genes (Beclin-1, ATG5, ATG12, ATG7) and proteins (Beclin-1, LC3) respectively. Data shown are means ± SD (n = 3, *P<0.05, compared with the control). β-actin was used as an internal control. E. Immunoblot assay for the PI3K-AKT-mTOR pathway.

treated with RA using an inverted phase contrast microscope showed that the amount of cells treated with RA gradually lessened, and the cells lost their tentacles to become spherical (**Figure 1A**).

RA-induced autophagy in HCT116 cells occurred via regulation of the PI3K-AKT-mTOR pathway

We observed that RA had a strong effect on the proliferation of HCT116 cells, but the mechanism was not yet clear. Our previous experiments suggested that RA inhibited the proliferation of gastric cancer cells by inducing apoptosis and autophagy. We wanted to investigate whether RA could induce autophagy in HCT116 cells and to this end we took TEM scans of the cells. The TEM results showed autophagosomes in the cytoplasm (**Figure 2A** and **2B**), which are indicative of autophagy. For further evidence, we performed RT-PCR and WB. The results of RT-PCR showed that the expression of the autophagy-related genes Beclin-1, ATG5,

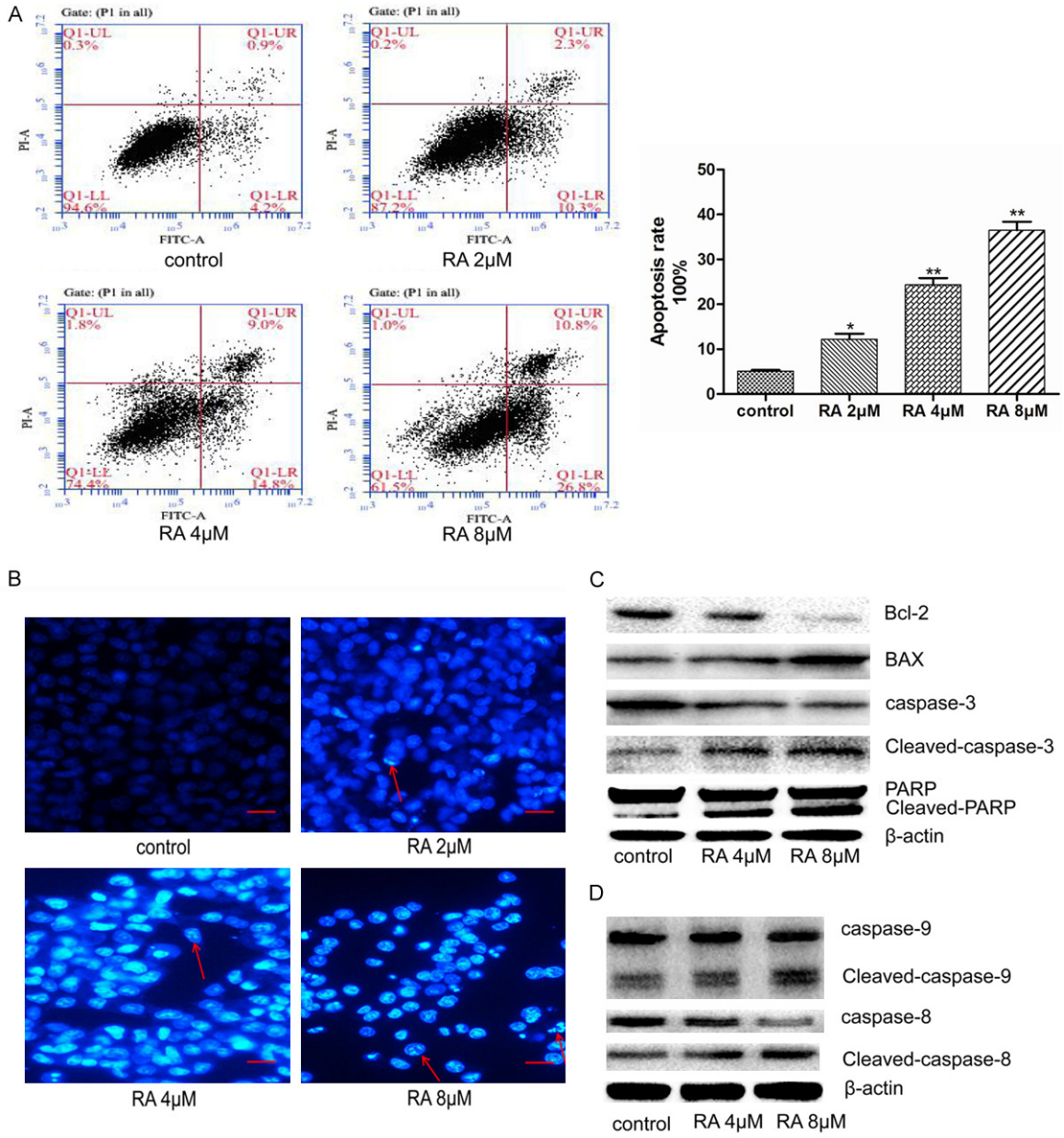

Figure 3. Raddeanin A (RA) induced apoptosis in HCT116 cells via the intrinsic and extrinsic pathways. A. After being treated with RA (2, 4, 8 μM) for 12 h, cells were incubated with Annexin V-FITC and propidium iodide (PI), and the apoptosis rate was analyzed via flow cytometry. Results shown are of an experiment representative of apoptosis. Q1-UL represents necrotic cells, Q1-UR represents cells at a later stage of apoptosis, Q1-LL represents viable cells, Q1-LR represents cells were at an early stage of apoptosis. Data shown are means ± SD (n = 3, *P<0.05, **P<0.01, compared with the control). B. Apoptotic cells were stained with Hoechst 33258 (50 ng/mL) after treatment with RA (2, 4, 8 μM) for 12 h, which was recorded with a Zeiss Axioplan 2 fluorescence microscope (400×). C. Animmunoblot assay shows the expression of apoptosis related proteins (Bcl-2, BAX, caspase-3, cleaved-caspase-3, cleaved-PARP and PARP). D. Cells were treated with RA and levels of apoptosis-related proteins (caspase-9, caspase-8) were determined via western blot. β-actin was used as an internal control. Bar = 50 μm.

ATG12, and ATG7 increased with increasing concentrations of RA (**Figure 2C**). WB indicated that the expression of Beclin-1 increased with RA treatment, and that with an increase in RA concentration, the signal intensity of the protein LC3I decreased while LC3II levels increased (**Figure 2D**). Furthermore, the classic signaling pathway for autophagy regulation is the PI3K-AKT-mTOR pathways. Western blot results also showed downregulation of p-AKT and p-mTOR proteins along with upregulation of PI3K, AKT, and mTOR proteins (**Figure 2E**).

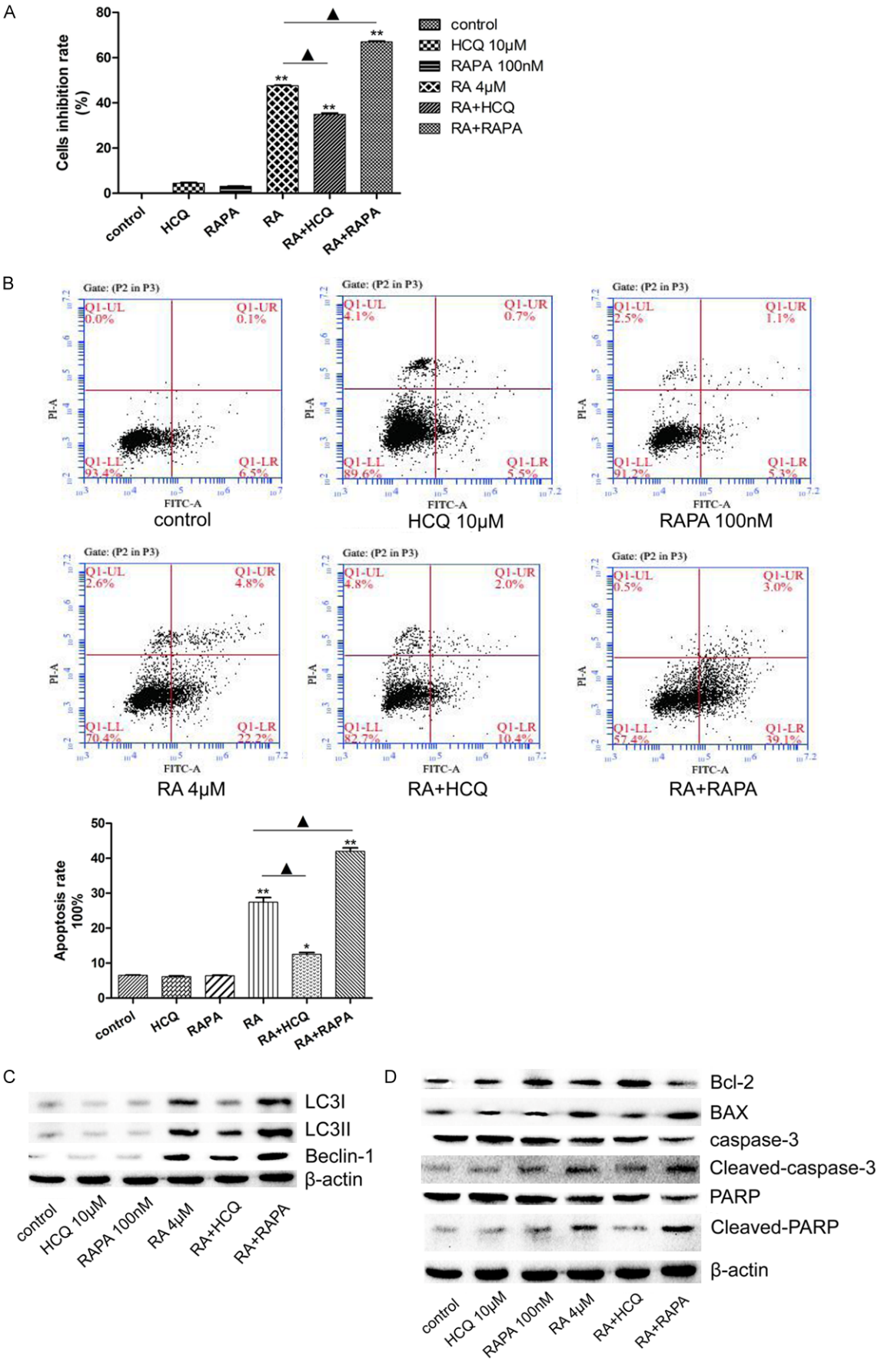
A
Cells inhibition rate (%)
control
HCQ 10μM
RAPA 100nM
RA 4μM
RA+HCQ
RA+RAPA
B
Gate: (P2 in P3)
Q1-UL 0.0%
Q1-UR 0.1%
Q1-LL 93.4%
Q1-LR 6.5%
PI-A
FITC-A
control
Q1-UL 4.1%
Q1-UR 0.7%
Q1-LL 89.6%
Q1-LR 5.5%
HCQ 10μM
Q1-UL 2.5%
Q1-UR 1.1%
Q1-LL 91.2%
Q1-LR 5.3%
RAPA 100nM
Q1-UL 2.6%
Q1-UR 4.8%
Q1-LL 70.4%
Q1-LR 22.2%
RA 4μM
Q1-UL 4.8%
Q1-UR 2.0%
Q1-LL 82.7%
Q1-LR 10.4%
RA+HCQ
Q1-UL 0.5%
Q1-UR 3.0%
Q1-LL 57.4%
Q1-LR 39.1%
RA+RAPA
Apoptosis rate 100%
C
LC3I
LC3II
Beclin-1
β-actin
control
HCQ 10μM
RAPA 100nM
RA 4μM
RA+HCQ
RA+RAPA
D
Bcl-2
BAX
caspase-3
Cleaved-caspase-3
PARP
Cleaved-PARP
β-actin

Figure 4. Autophagy induced by Raddeanin A (RA) in HCT116 cells promote apoptosis. A. Prior to treatment with dealt with RA (4 μM), cells were treated with either hydroxychloroquine (HCQ; autophagy inhibitor, 10 μM) or RAPA (autophagy agonist, 100 nM) for 1 h. An MTT assay indicated different rates of cellular inhibition for the different groups. Data shown are means ± SD (n = 3, **P<0.01, compared with the control; ▲P<0.05, compared with the RA group). B. Cells were incubated with Annexin V-FITC and propidium iodide (PI), and the apoptosis rate was analyzed via flow cytometry. Q1-UL represents necrotic cells, Q1-UR represents cells at a later stage of apoptosis, Q1-LL represents viable cells, and Q1-LR represents cells at an early stage of apoptosis. Data shown are means ± SD (n = 3, *P<0.05, **P<0.01, compared with the control; ▲P<0.05, compared with the RA group). C and D. Western blot analysis showed the levels of autophagy and apoptosis related proteins. Protein levels of Beclin-1, LC3, Bcl-2, BAX, caspase-3, and PARP were determined via western blotting. β-actin was used as an internal control.

RA induced apoptosis in HCT116 cells through both intrinsic and extrinsic pathways

The lack of a working apoptosis mechanism is one of the main reasons for the formation of tumors. In this experiment, the MTT assay showed that RA could significantly inhibit the proliferation of HCT116 cells, so we hypothesized that RA could induce apoptosis in HCT116 cells. The results of the flow cytometry illustrated that the higher the concentration of RA, the higher the apoptosis rate (**Figure 3A**). Hoechst 33258 Staining also confirmed the existence of apoptosis, since we found the broken pieces of the nucleus which are a major symbol of apoptosis (**Figure 3B**). Western blot results also revealed that the expression of the proteins cleaved-caspase-3, cleaved-PARP, and BAX increased while the expression of the proteins Bcl-2, caspase-3, and PARP decreased (**Figure 3C**). Caspases-8 and 9, proteins critical to the intrinsic and extrinsic pathways, respectively, were also upregulated (**Figure 3D**).

The relationship between autophagy and apoptosis induced by RA in HCT116 cells

More and more data show that there are complex relationships between autophagy and apoptosis. Hydroxychloroquine (HCQ), which inhibits autophagy, and rapamycin (RAPA), an autophagy agonist, were added to the HCT116 cells along with RA. As shown in **Figure 4A**, when treated with HCQ (10 μM) and RA (4 μM) together, the cellular inhibition decreased compared with RA (4 μM) alone. According to the flow cytometry results, the apoptosis rate of the RA group (4 μM) was higher than that of the HCQ/RA combined group, but was lower than that of the RAPA (100 nM) and RA (4 μM) combined group (**Figure 4B**). The WB results also showed that the density of Beclin-1, LC3, cleaved-caspase-3, cleaved-PARP, BAX increased in the RA/RAPA group (**Figure 4C** and **4D**).

Discussion

A survey found that with a change in diet, the incidence of colorectal cancer has significantly increased in developing countries, and that patients tend to be younger [9]. Chemotherapy is the main treatment method for patients who have no opportunity for surgery [10]. However, with the increase in modern chemotherapy drugs and the frequency of use and length of treatment, cancer cells are no longer as sensitive to chemotherapeutic drugs. Thus, newantitumor drugs must be found as soon as possible.

RA, which is a type of natural medicine, has been shown to possess a significant anti-tumor effect both in vivo and in vitro [11-14], although the mechanism is unclear.

As the MTT assay showed, RA could inhibit the growth of HCT116 cells even at low concentrations (1 μM), and the inhibition rate was positively correlation with both RA concentration and incubation time (**Figure 1**). We hypothesized that this inhibition might be caused by the induction of autophagy and apoptosis. The involvement of autophagy inhibition is still controversial in tumor research. This is because while autophagy can lead to cell death, it also can protect cells from death in the case of hunger or loss of energy, and makes cells steady by removing damaged organelles, such as mitochondria, endoplasmic reticulum, and peroxidases [15].

To verify RA-induce autophagy in HCT116 cells, we observed the cells using TEM, and found the existence of a spherical double layer membrane, the autophagosome, considered to be the golden standard of autophagy determination (**Figure 2A** and **2B**). Moreover, the results of RT-PCR and WB also showed the upregulation of related genes (Beclin-1, ATG5, ATG7, ATG12) and pro-autophagy proteins. As is

shown in **Figure 2D**, with increasing concentrations of RA, LC3I converted to LC3II, which is required to form the autophagosome membrane. Previous reports have suggested that PI3K-AKT-mTOR is the major autophagy signaling. The Nobel Prize winner Ohsumi also confirmed that the PI3K complex is the key to the process of autophagy [16]. Our experiments also found that the expression of p-AKT and p-mTOR decreased, whilethe expressions of PI3K, AKT and mTOR increased during autophagy, which supports the supposition that the PI3K-AKT-mTOR pathway is involved in regulating autophagy (**Figure 2E**).

With the exception of autophagy, induction of apoptosis the mainmechanism of most antitumor drugs, due to apoptosis being the earliest recognized process of tumor cell death. Apoptosis can be divided into three stages [17]: (1) Startup phase: Due to various causes, the cells start the process of apoptosis; (2) Effect phase: Whether apoptosis occurs is determined by the presence of apoptotic and antiapoptotic factors; (3) Execution phase: Apoptotic cells showed specific morphological and biochemical features, including DNA rupture, nuclearpyknosis, karyorrhexis, karyolysis, the formation of apoptosis bodies, and degradation by lysosomes. Flowcytometry and Hoechst 33258 staining showed that RA induced apoptosis in HCT116 cells, and that the rate of apoptosis increased with increasing concentrations of RA (**Figure 3A** and **3B**). In addition, the WB results also showed that fewer decreased expression of the anti-apoptotic protein Bcl-2 and increased expression of pro-apoptotic protein BAX (**Figure 3C**) after treatment with RA. As is well known, apoptosis has biochemical pathways, namely, the mitochondrial pathway, which is intrinsic pathway, the death receptor signaling pathway, which is extrinsic pathway, and the endoplasmic reticulum stress pathway. Activated caspase-9 is necessary for the intrinsic pathway to occur, while activated caspase-8 is necessary for the extrinsic pathway. In this study, we found that the expressions of caspase-8 and -9 both increased (**Figure 3D**), which revealed that RA induced apoptosis in HCT116 cells via intrinsic and extrinsic pathways.

Autophagy and apoptosis are both types of programmed cell deaths, but there are many differences between them in the cellular morphologies, molecular mechanisms, and biochemical indicators involved. It has been reported that in cells with a large number of autophagosomes, the use of caspase inhibitors does not affect cell death, while the autophagy inhibitor 3-methyl adenine does, which illustrates that the processes of autophagy and apoptosis are different [18]. Nonetheless, there may be a relationship between autophagy and apoptosis. Yee et al found that autophagy induced by PUMA and BAX could repair mitochondrial damage and reduce progress of apoptosis [19]. Hui L et al found that sodium selenite could promote cellular autophagy while suppressing apoptosis [20]. JingwenY found that 5-Fu combined with an autophagy or apoptosis inhibitor could promote HCT-116 cell proliferation and inhibit apoptosis, and that 5-Fu combined with autophagy inhibitor would produce the opposite result [21]. Moreover, under certain conditions, cells can transition between autophagy and apoptosis and use both processes to regulate tumor cell death. Forexample, when apoptosis was inhibited in HeLa and Chinese hamster ovary (CHO) cells, the cells died via autophagy instead [22]. In order to examine the relationship between RA-induced autophagy and apoptosis, the autophagy inhibitor HCQ and autophagy agonist RAPA were added to HCT116 cells treated with RA. As is shown in **Figure 4**, autophagy could promote RA-induced apoptosis in HCT116 cells.

In conclusion, RA can induce autophagy in HCT116 cells by regulating the PI3K-AKT-mTOR pathway and induce apoptosis via the intrinsic and extrinsic pathways. Moreover, autophagy can promote apoptosis, when induced by RA in HCT116 cells. These findings make RA a promising candidate for the treatment of colorectal cancer, and also lay the foundation for further clinical applications.

Acknowledgements

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Disclosure of conflict of interest

None.

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