Rapamycin induces human acute promyelocytic leukemia cell HL-60 autophagic apoptosis

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Abstract. – OBJECTIVE: We aimed at investigating the effects of rapamycin on apoptosis and autophagy of human acute promyelocytic leukemia cell line HL-60, and to preliminarily explore the mechanism of extra medullary infiltration of leukemia cells with human acute promyelocytic leukemia cell line HL-60 as the object of study, providing a theoretical basis for the clinical treatment of leukemia.
MATERIALS AND METHODS: After HL-60 cells were cultured *in vitro*, the effect of rapamycin on proliferation ability of HL-60 cells was determined by methyl thiazolyl tetrazolium (MTT) method, the cell apoptosis ratio was detected by flow cytometer, the change of autophagy after HL-60 cells acted by rapamycin was tested by monodansylcadaverine (MDC) fluorescence staining, the mRNA expression of autophagy-related molecule was detected by polymerase chain reaction (PCR), and the expressions of apoptosis-related protein and autophagy-related protein were determined by Western blotting (WB).
RESULTS: HL-60 cell proliferation could be significantly inhibited by rapamycin (80 μg/mL-640 μg/mL), which was in a dose-dependent manner. HL-60 cell apoptosis ratio and apoptosis-related protein expression were distinctly improved by rapamycin. Cell autophagy level, mRNA expression of autophagy-related molecule and autophagy-related protein expression were remarkably induced by rapamycin.
CONCLUSIONS: Rapamycin can induce HL-60 cell apoptosis, which is produced mainly by inducing cell autophagy.

Key Words:
Rapamycin, HL-60, Cell apoptosis, Cell autophagy.

Introduction

Leukemia is a kind of malignant proliferative disease of hematopoietic system, which incidence rate is increasing day by day[1,2]. Combined chemotherapy is still the main treatment for leukemia. However, there are many problems in the treatment process, such as serious complications and multidrug resistance. Moreover, toxic and side effects of chemotherapy drugs also restrict the treatment effect of drugs[3]. Therefore, how to choose the safe and effective drugs has always been an important research topic. Rapamycin (RAPA) belongs to macrolides, which is essentially an antibiotic and an inhibitor of the mechanistic/mammalian target of rapamycin (mTOR) pathway. As an immunosuppressant, rapamycin inhibits growth and proliferation of immune cells via blocking signal transduction. It exerts the effect of immunity suppression through inhibiting the synthesis of Interleukin-2 (IL-2) and other immune molecules, and restrains the production of IgG in patients with transplantation and the occurrence of donor-specific immunity[4,5]. Compared with the traditional immunosuppressive agents, rapamycin is characterized by the small dosage, high efficiency and small toxic side effects, especially small renal toxicity. Studies have shown that rapamycin has a dual effect, namely immunosuppressive effect and anti-tumor effect. It inhibits intracellular cycle conversion and intracellular DNA replication

through inhibiting mTOR-signaling pathway; as a result, the cells are failed to perform normal mitosis. Rapamycin can induce tumor cell apoptosis and inhibit angiogenesis, tumor invasion and vascular endothelial proliferation, so as to exert anti-tumor effect[6-8], which is confirmed by the experimental investigation. Currently, rapamycin has been utilized in the treatment of some solid tumors in clinical practice, achieving a certain effect. Experiments have shown that rapamycin exerts the role primarily by inhibiting mTOR-signaling pathway. Thus, the effect of rapamycin in the treatment of hematopoietic malignancies is attracting more and more attention and investigation. Previous studies on the mechanism of anti-tumor drugs focused on blocking the cycles of tumor cells and surrounding vascular endothelial cells, reducing the tumor cell infiltration, and inducing the apoptosis and necrosis, etc. With the constant deepening of research on autophagy, the roles of autophagy in tumor occurrence, development, and extinction, have attracted more attention[9,10]. The present work[11] have found that there are changes in autophagic activity in a variety of human tumors. Autophagy is considered to be the mode of type II programmed cell death, which plays a pivotal role in cell metabolism like apoptosis[12], involved in tumorigenesis, neurodegeneration, microbial infection, aging and other pathophysiological processes. Therefore, in this study, the effects of rapamycin on apoptosis and autophagy of HL-60 cells were investigated through *in vitro* experiments with HL-60 cells, so as to provide an experimental basis for searching new chemotherapy drugs of leukemia.

Materials and Methods

Cells and Experimental Materials

Human acute promyelocytic leukemia HL-60 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Rapamycin was purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies caspase3 and cleaved-caspase3 were purchased from CST (Danvers, MA, USA), and the secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); serum-free cell freezing medium Roswell Park Memorial Institute-1640 (RPMI-1640) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA); flow cytometry kit, methyl thiazolyl tetrazolium (MTT) kit and Cytotoxicity Detection Kit (LDH) were purchased from Nanjing Keygen Biotech. Co., Ltd. (Nanjing, China).

Cell Culture

Human acute promyelocytic leukemia cells (HL-60 cells) were cultured on the Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS at 37°C in an incubator with saturated humidity and 5% CO_2. Under the adherent growth, the cell concentration was adjusted to be 5×10^5 cells/mL after the cell covered the bottle bottom (usually after 2-3 d), followed by 0.25% trypsin digestion and passage under sterile conditions.

MTT Assay

Cells in the logarithmic growth phase were digested and collected, prepared into the cell suspension with the concentration of 1×10^5 cells/mL, and inoculated onto the 96-well plate (100 µL/well). Three control wells and the blank control were set. After inoculation overnight, the cells were well adhered confirmed via microscopic observation. Diluted rapamycin was added and the final concentration was 0 µM, 5 µM, 10 µM, 20 µM, 40 µM, 80 µM, 100 µM, 160 µM, 320 µM and 640 µM, in turn. The 0 µM group was the control of RPMI-1640 medium. Cells were incubated at 37°C in an incubator with saturated humidity and 5% CO_2 for 24 h. 4 h before the end of incubation, 20 µL MTT was added into each well, followed by incubation at 37°C for 4 h. Then, the supernatant was carefully absorbed, and 150 µL dimethyl sulfoxide (DMSO) was added into each well, followed by vibration for 10 min. The optical density (OD value) of each well at the wavelength of 630 nm was detected using the microplate reader. The experiment was repeated three times. Proliferation inhibition was calculated as follows: inhibition rate = OD value of drug group/OD value of control group ×100%.

Flow Cytometry

HL-60 cells were inoculated onto the 6-well plate (5×10^5 cells/mL) overnight, and the rapamycin solution with the final concentration of 0 µM, 80 µM and 160 µM was added for incubation at 37°C in an incubator with saturated humidity and 5% CO_2 for 24 h, respectively,

followed by trypsin digestion. Then, cells were centrifuged at 1000 g/min for 4 min at 4°C and they were collected; the medium was discarded. The centrifuged cells were washed twice with cold polybutylene succinate (PBS). Next, cells were suspended using 200 µL Binding Buffer at a concentration of about l×l0⁶ cells/mL. 10 µL Annexin V-Fluorescein isothiocyanate (FITC) was added into the cell suspension and incubated for 15 min at room temperature in a dark place, and 5 µL propidium iodide (PI) was added and mixed gently. The detection was performed within 1 h with flow cytometer. Data were analyzed using CellQuest software. Each experiment was repeated for three times.

Monodansylcadaverine (MDC) Staining

HL-60 cells in the logarithmic growth phase were taken and the cell density of each group was adjusted to be 10×10⁶ cells/mL. The cells were inoculated onto the 6-well plate and three control wells were set in each group. 5 mL culture solution containing cells was added into each well for incubation at 5% CO_2 at 37°C under saturated humidity for 5-6 h. Then, 0.5 mL rapamycin solution with the final concentration of 0 µM, 80 µM and 160 µM was added for incubation for another 24 h. Cells were fixed with methyl alcohol at room temperature for 10-15 min, added with 2 mL 0.05% Triton-x100 phosphate buffer saline (PBS) solution and placed at room temperature for 10 min. Each group was equally divided into two pieces and inoculated onto the 24-well plate (1 mL/well) for incubation. 0.5 µL 0.1 M MDC fluorescent staining was added into each well and the final concentration was set as 50 µM. Then, it was coated with tinfoil and placed in an incubator with 5% CO_2 and saturated humidity for 60 min at 37°C. Next, cells were centrifuged at 800 rpm for 10 min and they were collected. They were detected using the flow cytometer and the proportion of MDC positive cells was detected using the CellQuest software. A small number of cells was resuspended and coated on the glass slide, covered with cover glass, followed by observation immediately under the inverted fluorescence microscope with the excitation wavelength of 450-490 nm, and photographed.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

HL-60 cells treated by different concentrations of drug in the treatment group and control group were collected and transferred into 1.5 mL ribozyme-free Eppendorf (EP) tube, followed by being added with 1 mL TRIzol and uniformly beat upon until solution becoming clear, making the cells fully denatured. 200 µL trichloromethane was added, and the sample was shaken vigorously and mixed well, followed by being placed on the ice for 15 min. The cells were centrifugated at 12000 rpm at 4°C for 15 min. The supernatant was removed, and 500 mL dimethyl carbinol was added, mixing well, followed by being placed on the ice and centrifuging at 12000 rpm at 4°C for 10 min. The supernatant was discarded, and 75% alcohol was added to rinse the precipitation, followed by centrifuging at 8000 rpm at 4°C for 5 min. The supernatant was discarded, the precipitation was dried and dissolved in 20 µL diethyl pyrocarbonate (DEPC) water, and the sample concentration was measured. The extracted RNA was reversely transcribed. After cDNA reaction system was synthesized, the conditions of reverse transcription were set to perform PCR amplification. After each cycle, the fluorescence signal was collected in Real-time, and the amplification and dissolution curves were recorded.

Western Blotting

The cells in each group were taken and washed twice with D-Hank's, which was cleared by absorbent paper. 150 µL pre-cooled Lysis Buffer was added into each group, followed by splitting on ice for 30 min. Proteins in each group were collected using the cell scraper and placed into the EP tube, followed by centrifugation at 4°C at 12000 rpm. The supernatant was taken and transferred to a new EP tube. After bicinchoninic acid (BCA) method was used to detect the protein concentration, 5×loading buffer was added and mixed well, and the protein was heated at 100°C for 6 min. 30 µL protein was added into the prepared loading wells of separation gel and concentrated gel, followed by electrophoresis in electrophoretic buffer solution at a suitable voltage. After electrophoresis, the gel was adhered closely to polyvinylidene difluoride (PVDF) membrane, followed by membrane transfer in transfer buffer at 0°C under the constant voltage of 100 V for 60 min. After the PVDF membrane was sealed at room temperature in 5% skim milk powder for 1 h, the PVDF membrane was cut according to the molecular

weight and sealed with the primary antibody in the refrigerator at 4°C overnight. On the next day, the PVDF membrane was rinsed in Tris buffered saline with Tween-20 (TBST20) and anti-IgG second antibody (1:5000) was added for incubation at room temperature for 1 h. After incubation, the membrane was rinsed again with TBST, followed by color development using Tannon 5200 fluorescence immunoassay development system and gray level calculation.

Statistical Analysis

The experimental data were presented as mean ± standard deviation (x±s), and statistical treatment was performed using Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for intergroup comparison or *t*-test was applied for comparisons between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Rapamycin Inhibits Proliferation of HL-60 Cells

The effect of rapamycin in different concentrations on cell proliferation was detected by MTT method. The results displayed that rapamycin in a certain concentration could inhibit cell proliferation compared with that in control group. The inhibition rate of cell proliferation in the 80 μM group was 25±2.5%, which was 40±1.6% in the 160 μM group, 75±2.3% in the 320 μM group, and 100±1.9% in the 640 μM group. The difference in inhibition rate between each group of 80 μM, 160 μM, 320 μM, 640 μM and control group was obvious, and there were differences between the groups, indicating that the proliferation of HL-60 cell inhibited by rapamycin was in a dose-dependent manner (Table I).

Rapamycin Induces Apoptosis of HL-60 Cells

The apoptosis was detected by flow cytometer after cells being acted by rapamycin in concentrations of 80 μM and 160 μM for 24 h. The early and late apoptosis rate in the 0 μM group were 1.04% and 0.38%, respectively, and

Table I. Effect of rapamycin on the inhibition rate of HL-60 cell proliferation.

Drug concentration (μM)	Inhibition rate (x̄±s)
0	0
5	16±1.4%
10	19±2.1%
20	25±2.5%
40	31±2.3%
80	40±1.6%[*]
100	75±2.3%[**]
160	82±1.8%
320	91±2.2%
640	99±0.8%[***]

[*]Compared with 0 concentration, p<0.05; [**]Compared with 80 μM, $p < 0.05$; [***]Compared with 160 μM, $p < 0.05$.

the total apoptosis rate was 1.12%. The early and late apoptosis rate in the 80 μM group were 25.4% and 4.87%, respectively, and the total apoptosis rate was 30.27%. The early and late apoptosis rate in the 160 μM group were 38.49% and 9.03%, respectively, and the total apoptosis rate was 48.52% (Figure 1A). Statistics showed that the ratio of early and late apoptosis cells in HL-60 cell apoptosis induced by rapamycin was increased with the increase of concentration of drugs (Figure 1B). The expression of apoptosis-related proteins was determined by Western blotting. The apoptosis-related proteins, caspase3 and cleaved-caspase3, were tested after cells being acted by rapamycin in concentrations of 80 μM and 160 μM for 24 h (Figure 2A). The results revealed that rapamycin remarkably increased the ratio of cleaved-caspase3 to caspase3, compared with that in the control group. With the increase in acting concentration of rapamycin, the expression of apoptosis-related protein was improved, demonstrating that cell apoptosis was induced and promoted by rapamycin, which was in a dose-dependent manner (Figure 2B-C).

Rapamycin Induces Autophagy of HL-60 Cells

The formation of autophagic corpuscles was detected by multidrug resistance (MDR). There was almost no fluorescence aggregation point in the control group, but the significant fluorescence aggregation points were observed in the two groups of cells that were activated by rapamycin in concentrations of 80 μM and 160 μM,
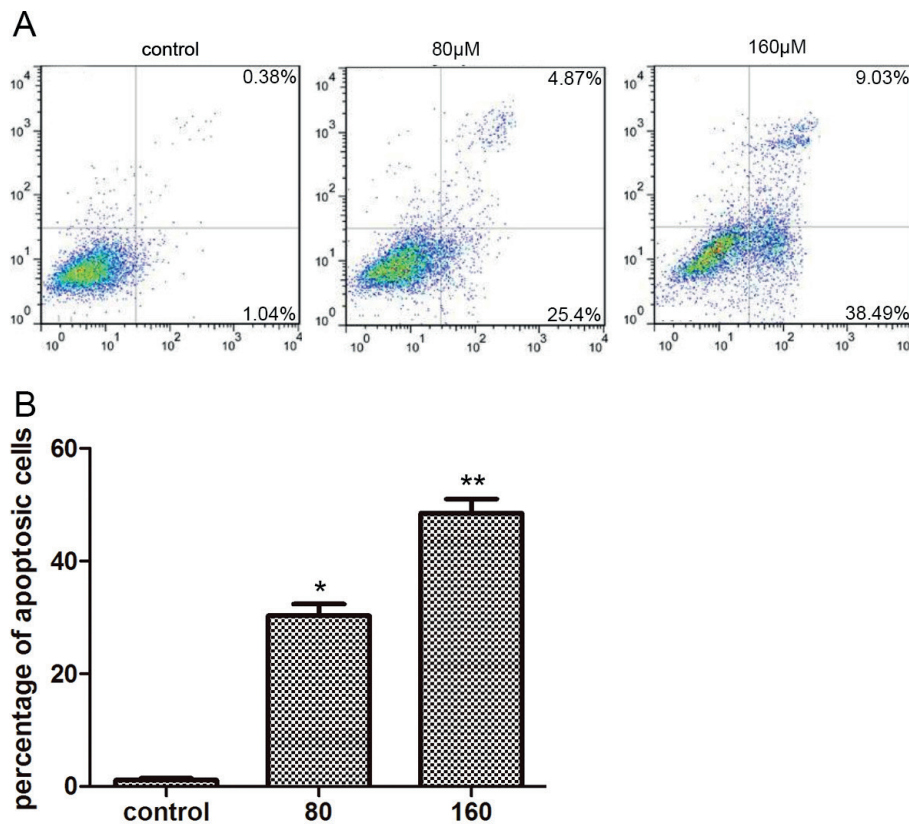
Figure 1. Detection of the percentage of apoptosis via flow cytometry. *(A)* Detection of cells apoptosis in the 0 μM, 80 μM, and 160 μM group via flow cytometry; *(B)* Detection of the total apoptotic cells in each group via flow cytometry. *Compared with the control group, $p<0.05$; **Compared with the 80 μM group, $p<0.05$.

indicating that the level of cell autophagy was evidently improved by rapamycin (Figure 3A). The detection using flow cytometer showed that the positive cell ratio in the control group was 4.43±1.22%, which was up to 28.76±2.14% in the 80 μM group, and 42.15±2.04% in the 160 μM group, indicating that autophagy cell ratio was significantly increased in the rapamycin group than that in the control group ($p<0.05$), which was in a dose-dependent manner (Figure 3B). The mRNA expression of autophagy-related molecule was detected by PCR. The mRNA expression of Atg5 molecule in cells of the two groups acted by rapamycin in concentrations of 80 μM and 160 μM was significantly increased than that of the control group, demonstrating that cell autophagy was distinctly increased at RNA level via acting with rapamycin (Figure 4A). The expressions of cell autophagy-related proteins, Atg5, LC3I and LC3II, were determined by Western blotting, displaying that

Atg5 protein expression and the ratio of LC3II to LC3I in cells of the two groups acted by rapamycin in concentrations of 80 μM and 160 μM were significantly increased than those of the control group (Figure 4B-D), suggesting that cell autophagy was distinctly increased at protein level via acting with rapamycin.

Discussion

Like the treatment of other malignant tumors, the traditional treatment of leukemia is killing leukemia cells to the lowest level and reducing the tumor load by chemotherapy to promote normal hematopoietic recovery. Whether there is a kind of drug that can induce tumor cell apoptosis but not damage, the normal tissues is investigated. Through the research, we found that the cell apoptosis ratio was increased with the unceasingly increase of concentration of rapamycin.

A
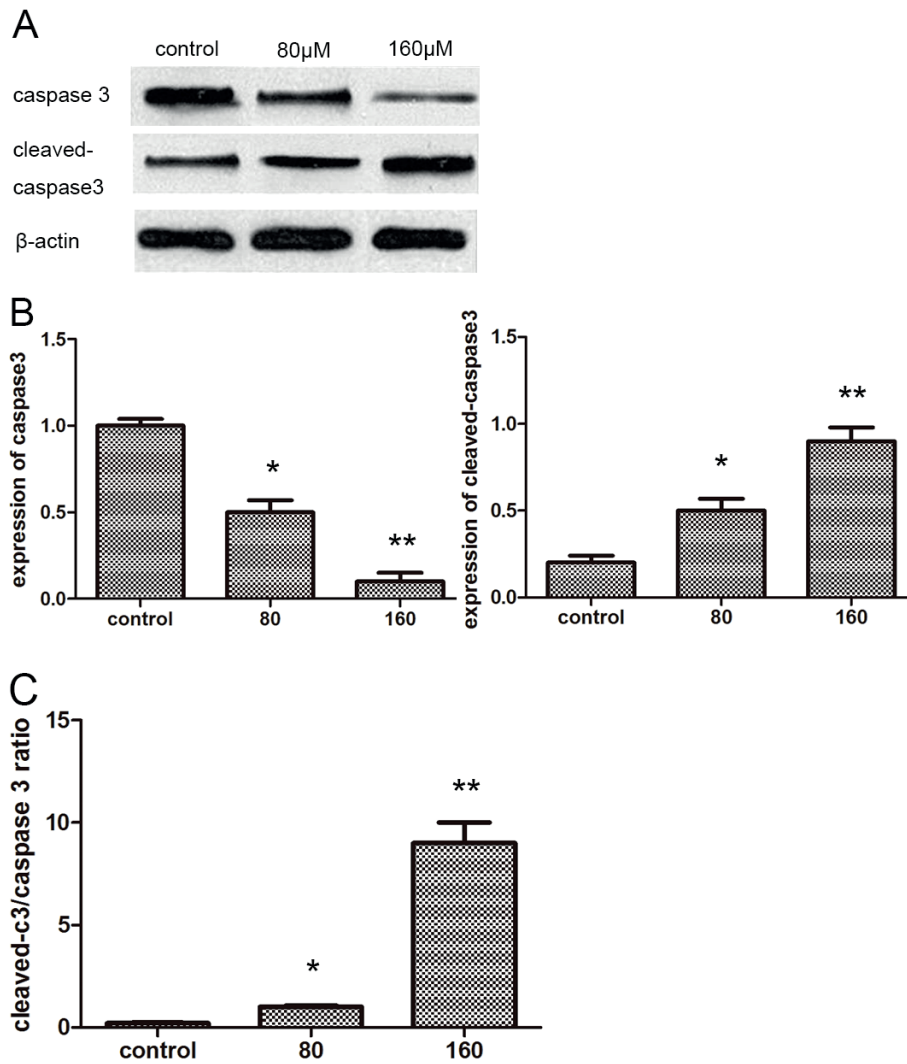
Figure 2. Detection of apoptosis via Western blotting. *(A)* Detection of the expressions of apoptosis-related proteins caspase3 and cleaved-caspase3 in the 0 μM, 80 μM and 160 μM group via Western blotting; *(B)* Gray scale scan and ratio analysis of caspase3 and cleaved-caspase3. *Compared with the control group, *p*<0.05; **Compared with the 80 μM group, *p*<0.05.

Rapamycin, an inhibitor of mTOR, is firstly applied as immunosuppressive agent in clinical practice and has achieved some results in rheumatic diseases. With the further study on the biological characteristics of rapamycin, it is also found that rapamycin has an anti-tumor property. In multiple studies on solid tumors, rapamycin has shown the ability to inhibit the growth of tumor cell activity, such as non-small cell lung cancer, prostate cancer, bladder cancer, ovarian cancer, breast cancer, gastric cancer, liver cancer, etc.[13-17]. Although there are no anti-tumor drugs to be produced mainly based on autophagy mechanism, the anti-tumor effect of those drugs is more or less correlated with autophagy[18]. Researches have found that there are changes in autophagic activity in a variety of human tumors[19]. Since the 1980s, people have gradually found that the autophagic capacity of tumor cells is weaker than that of surrounding normal cells, and it will not increase under the nutrition deficiency or high cell density. Therefore, it is proposed that the decreased autophagy function can improve the survival advantage of tumor cells[20,21]. Some tumor cells have weaker autophagic capacity after cancerization despite of different autophagic capacities before cancerization, suggesting
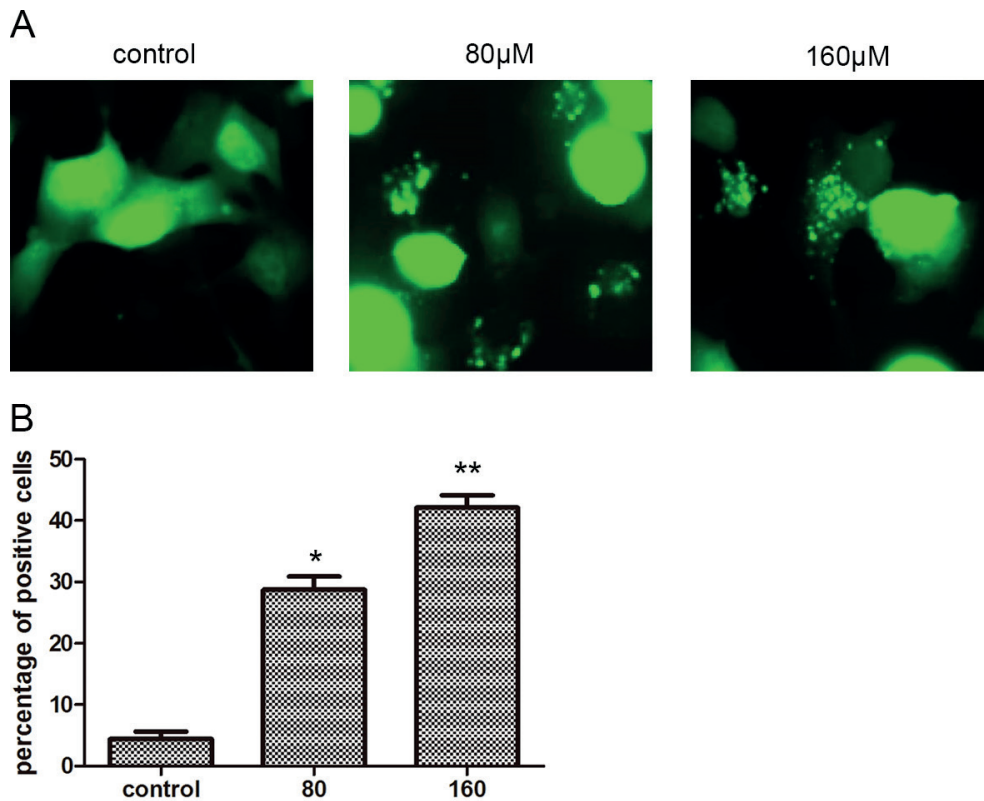
Figure 3. Detection of autophagosome formation via MDR. *(A)* Detection of LC3 green fluorescent protein aggregation in the 0 μM, 80 μM and 160 μM group via fluorescence microscopy (×1000); *(B)* Data analysis of green fluorescence aggregation cell ratio. *Compared with the control group, *p*<0.05; **Compared with the 80 μM group, *p*<0.05.

that the decline in autophagic capacity may be beneficial to the exacerbation of tumor[22,23]. Therefore, promoting the autophagic activity or even autophagic death of tumor cells may be a valuable new method of increasing the anti-tumor effect. MDC-positive cells were analyzed by flow cytometry in this study[24]. The percentage of cells with autophagic vesicles was detected to quantify the change of HL-60 cell autophagy ratio. In the experiment, after HL-60 cells were acted by 80 μM and 160 μM rapamycin for 24 h, they were followed by MDC fluorescence staining and observation under the fluorescence microscope. It could be seen that there was many apparently bright-green point-shape fluorescence, while the cells in the control group displayed the uniform greenish fluorescence. Meanwhile, through the detection of autophagy ratio in the experiment group by flow cytometer, we found that there was a statistically significant difference

between the experiment group and the control group (*p*<0.05), which was in a concentration-dependent manner. Subsequently, similar conclusions were obtained via detecting autophagy-related molecule mRNA and protein levels, indicating that rapamycin induces the autophagy of HL-60 cells.

Conclusions

Rapamycin can inhibit HL-60 cell proliferation and induce its apoptosis in a dose-dependent manner. The apoptosis-promoting effect may be produced by inducing cell autophagy.

Conflict of interest
The authors declare no conflicts of interest.
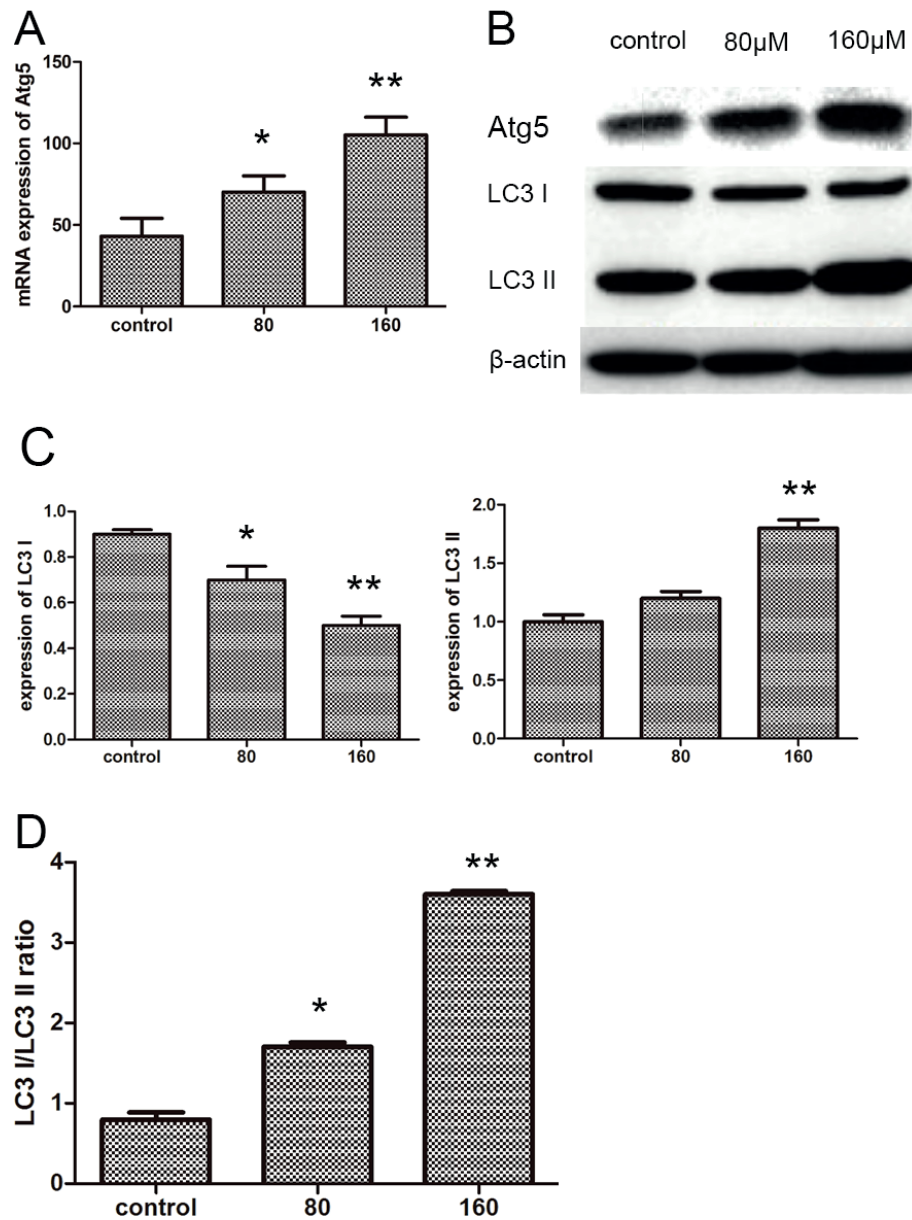
Figure 4. Detection of autophagy via PCR and Western blotting. *(A)* Detection of the mRNA expressions of autophagy-related genes Atg5 in the 0 μM, 80 μM and 160 μM group via PCR. *(B)* Detection of the expressions of autophagy-related proteins Atg5, LC3 I and LC3 II in the 0 μM, 80 μM and 160 μM group via Western blotting; *(C)* Gray scale scan and ratio analysis of LC3 I and LC3 II. *Compared with the control group, $p<0.05$; **Compared with the 80 μM group, $p<0.05$.

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