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ORIGINAL ARTICLE

Puerarin leads to K562 cell apoptosis of chronic myelogenous leukemia via induction of autophagy

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Summary

Purpose: *To study the effects of puerarin on the viability, apoptosis and autophagy of K562 cells of chronic myelogenous leukemia (CML), and to provide a basis for the study on antitumor mechanism of puerarin.*

Methods: *K562 cells of human CML were taken as the study material and puerarin was applied in different concentrations. The effect of puerarin on cell viability was detected via cholecystokinin-8 (CCK8) and lactate dehydrogenase (LDH). Flow cytometry and western blot (WB) were used to detect cell apoptosis, while Cyto-ID and WB were used to detect the cell autophagy level.*

Results: *Puerarin inhibited the K562 cell viability and increased cell apoptosis and autophagy in a dose-dependent manner. After 3-methyladenine (3-MA) autophagy inhibitor was used, puerarin's induction of cell autophagy was inhibited, and its apoptosis induction was also inhibited.*

Conclusions: *Puerarin increases the cell apoptosis through induction of autophagic apoptosis of K562 cells.*

Key words: *apoptosis, autophagy, chronic myelogenous leukemia, K562 cells, puerarin*

Introduction

Leukemia is a malignancy of human hematopoietic system and its incidence ranks sixth among all human malignancies. CML is an acquired hematopoietic stem cell clonal disease characterized by granulocyte's hyperplasia, and Philadelphia chromosome (Ph) is found in more than 95% of cells in bone marrow, which produce BCR-ABL fusion protein with a continuously-activated tyrosine kinase activity [1]. At present, chemotherapy is still the main treatment method of CML, but its side effects and drug resistance have become major obstacles to chemotherapy. Other emerging therapies, such as stem cell transplantation, have matching difficulties and other problems, so they cannot be used in the majority of patients. Previous studies on the mechanism of chemotherapeutic drugs focused on blocking the tumor cell cycle, inducing apoptosis and necrosis [2,3].

With the increasing knowledge on autophagy, its role in the development, progression and tumor cell death have attracted much attention [4,5]. Autophagy is a highly-conserved eukaryotic cell activity, mainly activated by nutritional deficiencies and other environmental stress stimuli, which degrade some organelles and longevity proteins in cells to provide essential nutrients for cells, ensuring the cell survival [6,7]. However, autophagy not only protects the cells, but also is often the reason of cell death caused by antitumor drugs [8]. Autophagic cell death is a kind of non-apoptotic programmed cell death [9], characterized by degradation of cellular contents via autophagosomes in dying cells. Therefore, in the drug therapy of CML, the drugs that can improve autophagic apoptosis of tumor cells can kill tumor cells and treat the diseases.

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Puerarin is a major active component isolated from the root of the traditional Chinese medical plant pueraria lobata. In recent years, it was found that extracts from pueraria lobata exert antioxidative effects, improve myocardial ischemia, reduce blood pressure and estrogens [10,11], while investigations on the mechanism of puerarin showed that it plays a regulatory role in cell autophagy. Recent studies have shown that extracts from pueraria lobata possess antitumor properties by inhibiting tumor cell proliferation and inducing tumor cell apoptosis in digestive tract tumors [12], hepatocellular carcinoma [13], and breast cancer [14]. A study on leukemia also found that puerarin has pro-apoptotic effect on acute lymphoblastic leukemia cells [15], but there has been no clear report yet on whether puerarin has pro-apoptotic effect on CML cells and whether its effect is realized by its regulation of cell autophagy.

The primary purpose of this study was to investigate the effect of puerarin on autophagy and apoptosis on CML cells.

Methods

Reagents and cell sources

Human CML cell line K562 was purchased from the cell bank of Chinese Academy of Sciences in Shanghai. Puerarin monomer was purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjin, China). Anti-Bcl2, anti-LC3B, anti-Bax, anti-Bcl2, anti-Caspase3 and anti-cleaved-Caspase3 were purchased from Cell Signal Technology (CST,USA), and 3-methyladenine (3-MA) was purchased from Selleckchem (Selleckchem, USA).

Cell culture and puerarin treatment

K562 cells were put in RPMI1640 culture solution containing 10% newborn bovine serum and 1% double antibody and cultured in an incubator containing 5% CO_2 at 37°C. The solution was replaced every other day and cells in the logarithmic growth phase were taken for the experiments. Puerarin was dissolved in DMSO in 100 mM concentration and diluted with cell culture medium when used. In cell culture medium, K562 cells were treated with puerarin with final concentrations of 50 μM and 100 μM for 24, 48 and 72 hrs, respectively.

CCK8 detection

Cell viability was detected on cells in the logarithmic growth phase that used for the CCK8 experiment (KeyGenBioTech, China); 0.25% trypsin and EDTA were used to digest cells and the supernatant was discarded after centrifugation. The cells were resuspended in DMEM containing 10% FBS. Then, the cells were counted and adjusted to 1.0×10^5 cells/mL and were inoculated onto the 96-well plate. Cell viability detection included the following steps: Cell suspension was inoculated onto the 96-well plate with 100 μL cells per well; then the cell suspension was cultured in an incubator containing 5% CO_2 at 37°C for 4 hrs to make cells adhere to the wall. Puerarin was given according to the experimental grouping with 4 wells per group. Ten μL CCK8 solution were added to each well of the 96-well plate and cultured in an incubator containing 5% CO_2 at 37°C for 4 hrs; the absorbance at 450 nm was measured using the microplate reader.

LDH detection

The cytotoxic effect was detected in cells in logarithmic growth phase via LDH (KeyGenBioTech,China); 0.25% trypsin and EDTA were used to digest cells. Then the cells were resuspended in DMEM containing 10% FBS. The cells were counted and diluted to 1.0×10^5 cells/mL, and then inoculated onto the 96-well plate with 5.0×10^3 cells/mL. Three control wells were set for each group, followed by cell adherence and puerarin treatment. The culture plate was incubated in an incubator containing 5% CO_2 at 37°C for 24 hrs. The corresponding reagents were added according to the kit instructions and the mixture was placed at room temperature for 3 min. The optical density (OD) value was measured by the microplate reader. 1 unit:1000 mL nutrient solution reacted with substrate for 15 min at 37°C, and 1 gmol pyruvic acid was produced in the system. LDH content in the culture medium was calculated according to the kit instructions.

Flow cytometry

The proportion of cell apoptosis was detected via flow cytometer (BD Biosciences, USA). Cells in logarithmic growth phase were digested and washed, and prepared into single-cell suspension; the cell density was adjusted into $(1\text{-}5)\times10^5$ cells/mL; 500 μL binding buffer were added to suspend and mix cells gently. After 5 μL Annexin-V-FITC was added, 5 μL propidium iodide (PI) were added and the suspension was mixed evenly; then the suspension was incubated at room temperature in the dark for 15 min. The experimental results were analyzed using BD CellQuest software.

Autophagy detection via Cyto-ID

The autophagy-related LC3 protein (ENZO) in cells was detected using Cyto-ID. Cells were grown on the coverslip and selected when the density was 50-70%, followed by intervention according to the experimental design; the negative control group was set up. The supernatant was discarded and the cells were washed twice with 1×Assay buffer for 30 min and were washed with 1×Assay buffer, and the excess buffer was removed, followed by sealing using anti-quenching agent containing 4'6-diamidino-2-phenylindole (DAPI) dye. Then, cells were incubated in the dark at 37°C . The coverslip was observed under fluorescence microscope (FITC and DAPI; 60×).

Western blotting

90-95% adherent cells were taken and the appropriate amount of 5× loading buffer was added; after

cells were collected on the ice using cell scraper and fully split in the ultrasonic pyrolyser, they were placed in the epoxy epoxide tube at 100°C phenyl methyl sulfonyl fluoride for water bath for 5-10 min, and 1× phenyl methyl sulfonyl fluoride (PMSF) was added in the final concentration. The concentration of cell protein was detected using bicinchoninic acid (BCA) method. The gel was prepared according to the configuration requirements of separation gel and spacer gel, the volume was calculated based on the protein concentration, and then the gel was added to each gel tank. After loading, the spacer gel was subjected to electrophoresis (80 V) and the separation gel was also subjected to electrophoresis (120 V). After protein electrophoresis, the gel block required was cut according to the molecular weight indicated in protein Marker, followed by membrane transfer on ice transfer buffer under voltage of 100 V for 1 hr. After that, the gel was sealed with 5% skim milk for 1 hr, and the primary antibody (anti-Bcl-2, anti-LC3, anti-Bax, anticaspase 3 and anti-cleaved caspase 3) was added for incubation at 4°C overnight. The next day, the gel was washed with PBS-T for three times and the secondary antibody anti-Bcl-2,anti-LC3, anti-Bax, anti-caspase 3 and anti-cleaved caspase 3) was added (1:10000;GE Healthcare, Little Chanfont, Buckinghamshire, UK) for incubation at room temperature for 1 hr; after washing three times, the color was developed using Tannon 5200 immunofluorescence development system and the gray scale was measured.

Statistics

SPSS13.0 statistical software was used. Data were presented as mean ± standard deviation; t-test was used for comparison between the groups, while one-way ANOVA was used for comparison among groups. P<0.05 suggested that the difference was statistically significant.

Results

Puerarin inhibited K562 cell proliferation

To investigate the effect of puerarin on the proliferation of K562 cells, CCK8 and LDH were used to detect cell viability and cytotoxicity. Puerarin with the final concentration of 50 μM and 100 μM was added into the K562 cell culture medium for 24, 48 and 72 hrs. CCK8 detection was performed for cells in the experimental group, the blank group (only medium was used) and the control group (the isopyknic solvent was added). The results showed that there was no significant difference in cell viability between control and experimental group, but the cell activity in the 50 μM puerarin group was slightly decreased, although without statistical difference compared with control group (Figure 1A). The cell viability of the 100 μM puerarin group was significantly decreased compared with the control group, and the cell activity was decreased by 50% after exposure for 48 hrs (Figure 1A). Similar results were found in the LDH detection of the supernatant. With increasing concentration and time of puerarin exposure, LDH leakage was increased and cytotoxicity was also increased (Figure 1B), while the cytotoxic effect was most significant after exposure to 100 μM for 48 hrs (Figure 1B).

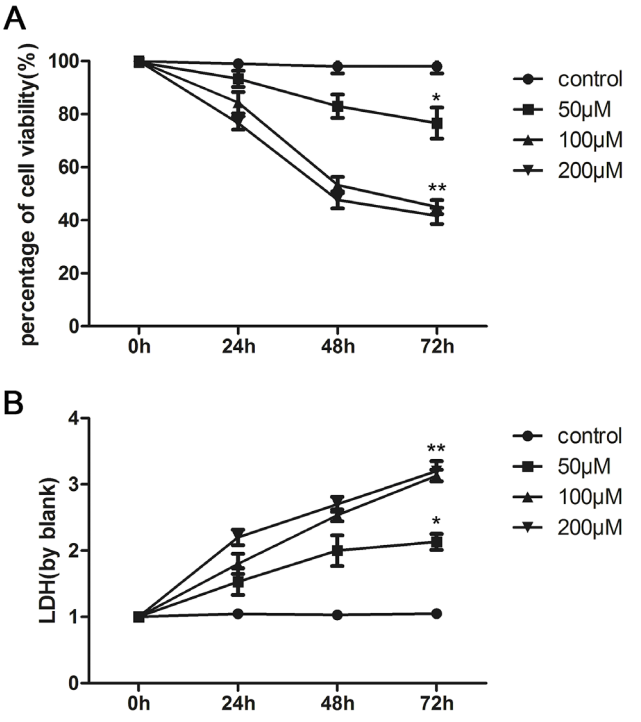

Figure 1. Cell viability at different concentrations and exposure times of puerarin. **(A)** Detection of percent cell viability at different concentrations and exposure times of puerarin via CCK8. **(B)** Detection of LDH leakage concentration at different concentrations and exposure times of puerarin via LDH assay (*compared with control group, p<0.05; **compared with control group, p<0.01).

Puerarin promoted K562 cell apoptosis

To study the effect of puerarin on K562 cell apoptosis, flow cytometry and WB were used. Flow cytometry was performed for cells treated with puerarin in different concentrations for 48 hrs and no significant difference between blank group and control group was noticed. The proportion of cell apoptosis was increased gradually with longer time of puerarin exposure. After 100 μM puerarin for 48 hrs, apoptosis reached about 52.78%, and the proportion of early apoptotic cells reached 46.9%, which was significantly increased compared with the control group (p<0.01) (Figure 2A,B). With continuous increasing exposure time of puerarin, the proportion of late apoptotic cells was increased, but that of early apoptotic cells showed no significant changes (Figure 2A,B). Similar results were noticed in the detection of apoptosis-related

proteins. Apoptosis was dose-dependent, and with the increased concentration of puerarin, the proportions of apoptosis-related proteins Bax/Bcl-2, cleaved-Caspase3/Caspase3, were gradually increased, which was most obvious in the 100 μM group ($p<0.05$) (Figure 2C,D).

Puerarin up-regulated K562 cell autophagy

To investigate the effect of puerarin on K562 cell autophagy, the formation of autophagosomes was observed via fluorescence microscopy after cell transfection using Cyto-ID LC3, and the autophagy-related proteins were detected. The results showed that there was no significant difference in autophagy fluorescence between the control and blank group. The autophagy was increased after 100 μM puerarin for 24 hrs, but no statistically significant difference compared with the control group was registered (Figure 3A,B). The autophagy fluorescence intensity was highest after 48 hrs ($p<0.05$) (Figure 3A,B). Similar results were found in the detection of autophagy-related proteins in each group. Atg5 expression and LC3II/LC3I ratio were increased with increasing time of exposure of puerarin, and the protein expression level was increased more significantly in the 48 hrs group than in the control group ($p<0.05$) (Figure 3C,D). According to the dose-effect relationship of puerarin of promoting cell apoptosis and autophagy, 100 μM puerarin for 48 hrs was used as the experiment condition of studying K562 cell apoptosis and autophagy.

3-MA inhibited puerarin-induced autophagy of K562 cells

To investigate whether the puerarin-induced apoptosis of K562 cells was related to autophagy, the autophagy inhibitor 3-MA (10 mM) was added to the cell culture medium treated with puerarin (100 μM) and the autophagy was observed after 48 hrs. The results showed that there was no significant difference in autophagy fluorescence between 3-MA and the control group, and the autophagy fluorescence in 3-MA+ puerarin group was significantly decreased compared with the puerarin alone group ($p<0.05$) (Figure 4A,B). In the detection of autophagy-related proteins it was found that the Atg5 expression and LC3II/LC3I ratio in the 3-MA group had no significant difference compared with those in the control group, and the expressions of autophagy-related proteins in 3-MA+ puerarin group were significantly decreased compared with those in the puerarin alone group ($p<0.05$). 3-MA significantly inhibited the puerarin-induced autophagy of K562 cells (Figure 4C,D).

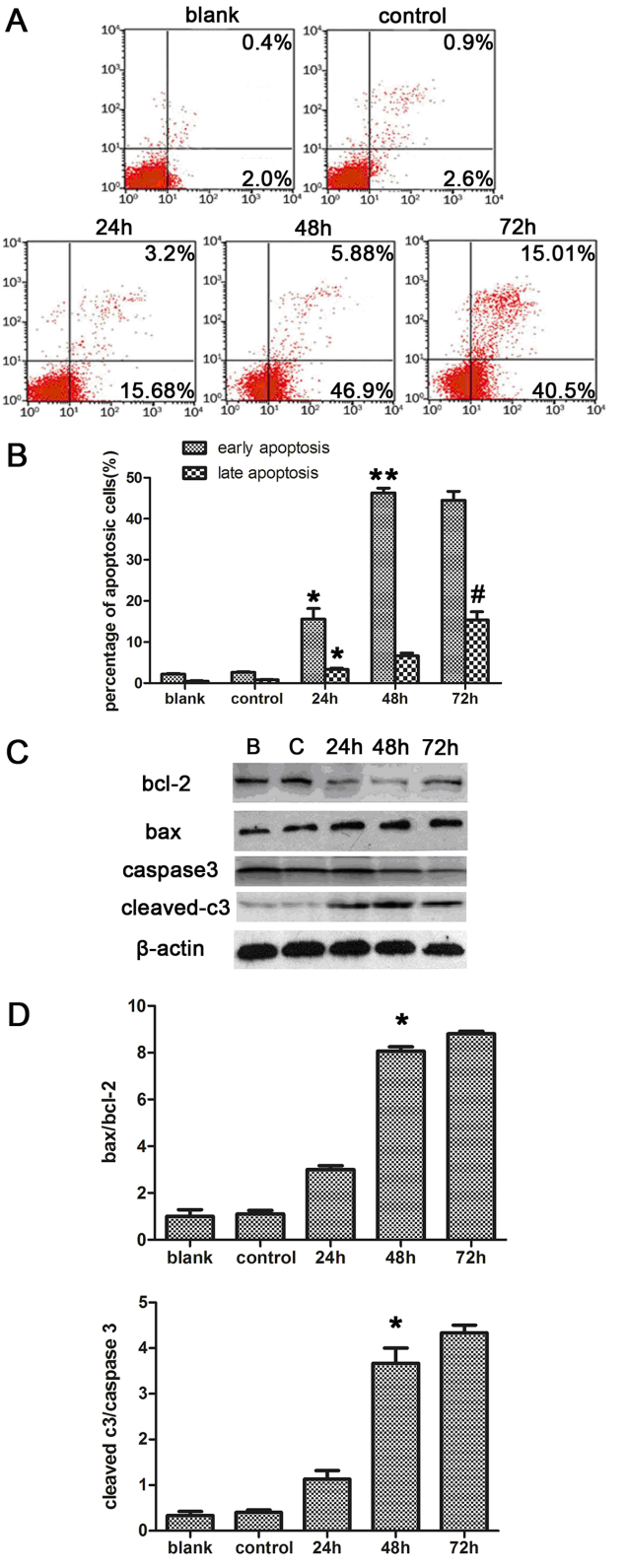

Figure 2. Cell apoptosis under different action time of puerarin (100μM). **(A)** Detection of proportion of cell apoptosis via flow cytometry. **(B)** Data statistics of proportions of early cell apoptosis and late cell apoptosis detected by flow cytometry. **(C)** Detection of concentrations of apoptosis-related proteins, Bcl-2, Bax, Caspase3 and cleaved-Caspase3, via Western blotting. **(D)** Scanning analysis of Bax/Bcl-2 and cleaved-Caspase3/Caspase3. (*compared with control group, $p<0.05$; **compared with the control group, $p<0.01$; #compared with the 48h group, $p<0.05$).

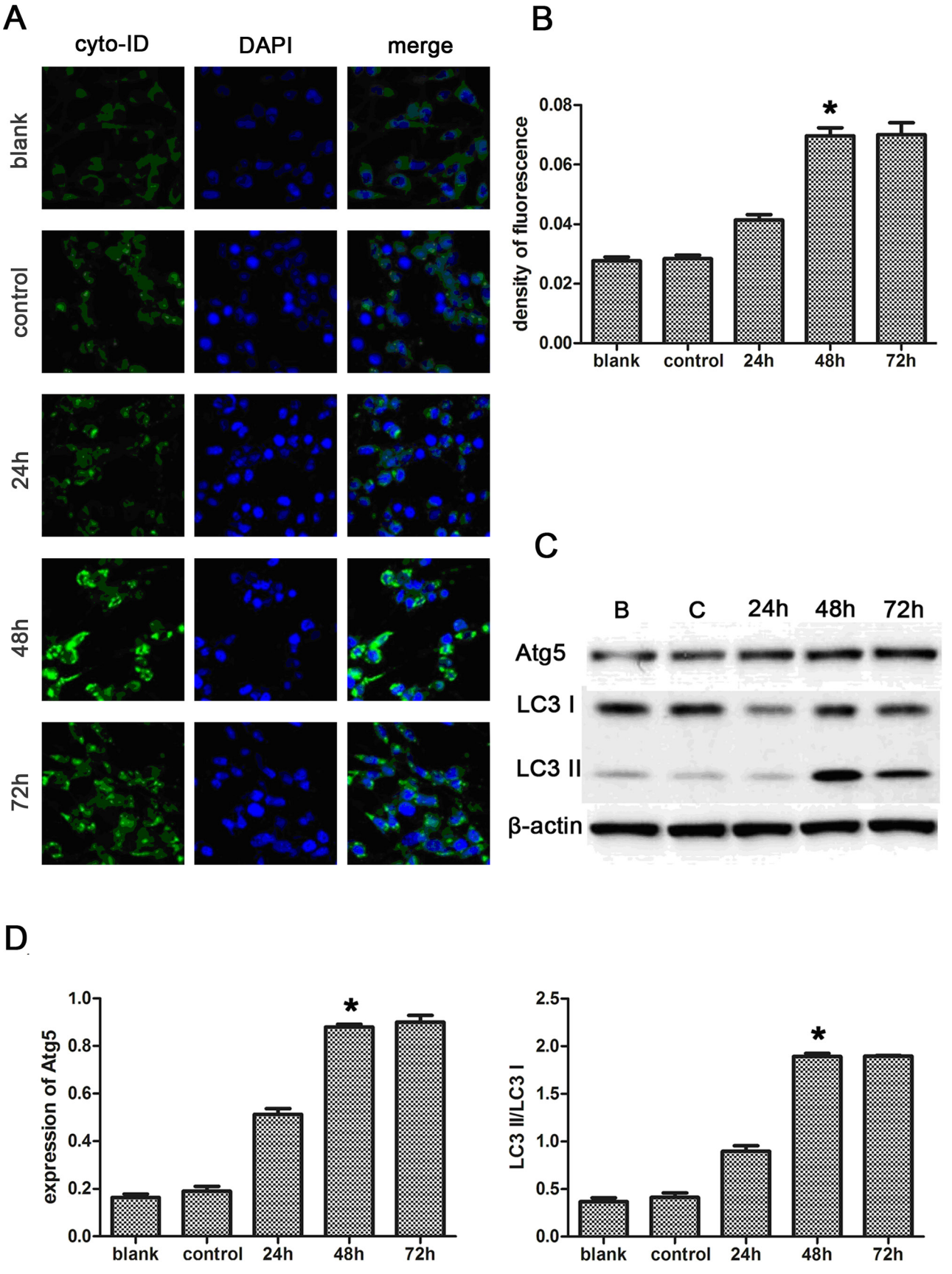

Figure 3. Cell autophagy under different action time of puerarin (100μM). **(A)** LC3 changes labeled by Cyto-ID fluorescence. **(B)** Data statistics of LC3 fluorescence intensity labeled by Cyto-ID. **(C)** Detection of concentrations of autophagy-related proteins, Atg5, LC3I and LC3II, via Western blotting. **(D)** Scanning analysis of Atg5 density and LC3II/LC3I ratio. (*compared with control group, $p<0.05$).

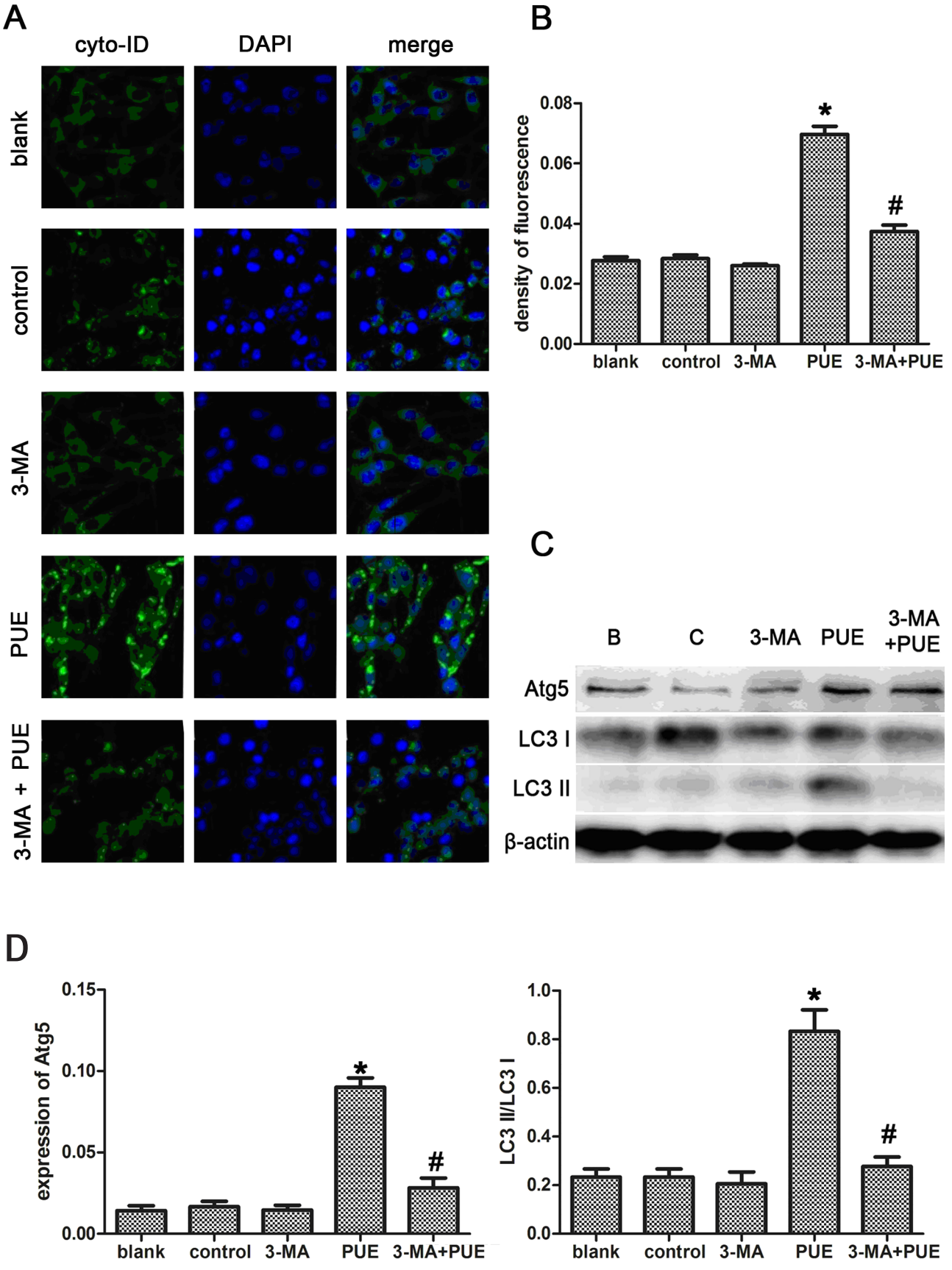

Figure 4. Cell autophagy after action of puerarin (100μM) and autophagy inhibitor 3-MA (10mM) for 48h. **(A)** LC3 changes labeled by Cyto-ID fluorescence. **(B)** Data statistics of LC3 fluorescence intensity labeled by Cyto-ID. **(C)** Detection of concentrations of autophagy-related proteins, Atg5, LC3I and LC3II, via Western blotting. **(D)** Scanning analysis of Atg5 density and LC3II/LC3I ratio. (*compared with the control group, p<0.05; # compared with puerarin group, p<0.05).

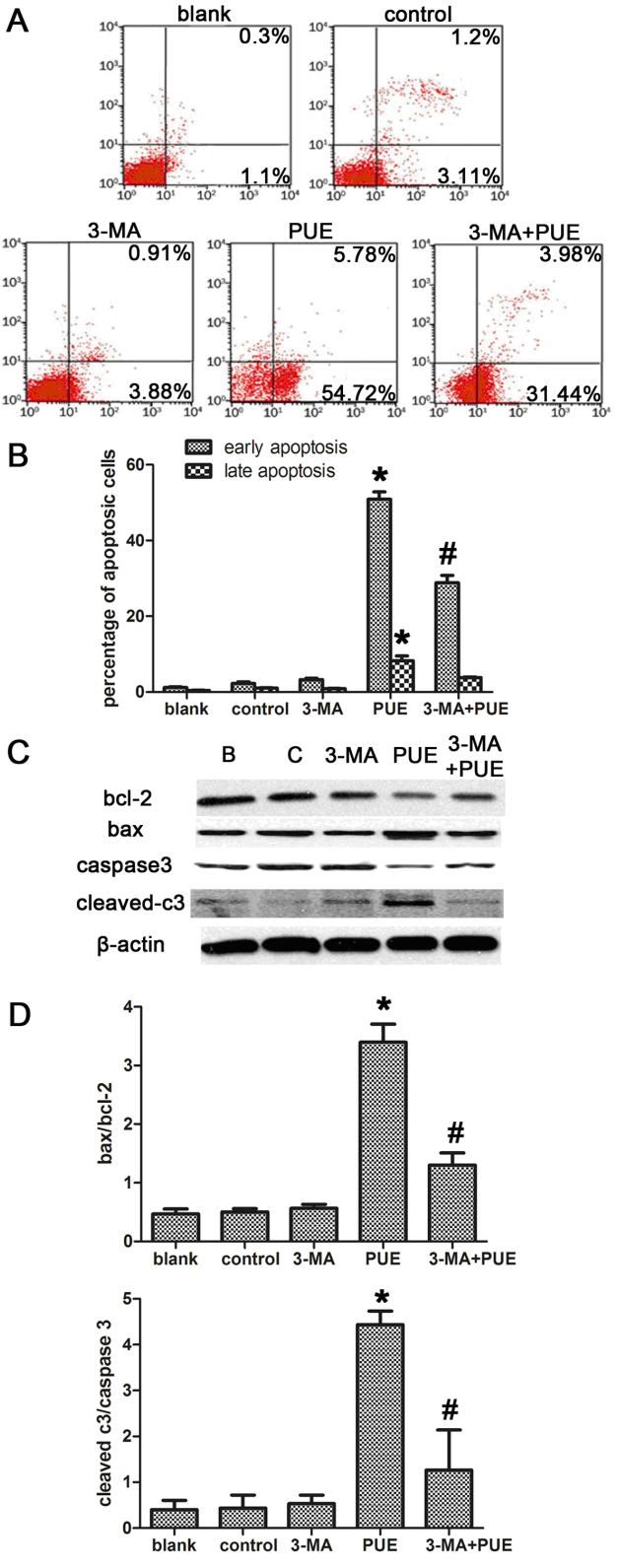

Figure 5. Cell apoptosis after action of puerarin (100μM) and autophagy inhibitor 3-MA (10mM) for 48h . **(A)** Detection of proportion of cell apoptosis via flow cytometry. **(B)** Data statistics of proportions of early cell apoptosis and late cell apoptosis detected by flow cytometry. **(C)** Detection of concentrations of apoptosis-related proteins, Bcl-2, Bax, Caspase3 and cleaved-Caspase3, via Western blotting. **(D)** Scanning analysis of Bax/Bcl-2 and cleaved-Caspase3/Caspase3. (*compared with the control group, p<0.05; #compared with the puerarin group, p<0.05).

3-MA inhibited puerarin-induced apoptosis of K562 cells

Autophagy inhibitor 3-MA (10 mM) was added to the cell culture medium treated with puerarin (100 μM) and apoptosis was observed after 48 hrs. The results showed that there was no significant difference in the proportion of apoptotic cells between 3-MA and the control group. The total proportion of apoptosis in 3-MA + puerarin group was 35.42% and the proportion of early apoptotic cells was 31.44%, showing significant decrease compared with those in the puerarin group ($p<0.05$) (Figure 5A,B). Similar results were noticed in the detection of apoptosis-related proteins. Compared with those in the control group, the expressions of apoptosis-related proteins Bax/Bcl-2 and cleaved-Caspase3/Caspase3 were not significantly different in the 3-MA group, but the expressions in 3-MA + puerarin group were significantly decreased compared with those in puerarin group ($p<0.05$) (Figure 5C,D).

Discussion

In this study, the effects of puerarin on viability, apoptosis and autophagy of K562 cells were investigated and analyzed. The results showed that puerarin significantly inhibited K562 cell viability exerting a clear cytotoxic effect. Flow cytometry, Cyto-ID LC3 and WB showed that puerarin promoted the apoptosis and autophagy of K562 cells in a dose-dependent manner, and the cell apoptosis was also decreased after the promoting effect of puerarin on autophagy was inhibited, proving that puerarin increases apoptosis by inducing autophagic apoptosis of K562 cells.

CML is one of the most common hematologic malignancies. At present, the main treatment methods are the traditional chemotherapy drugs or tyrosine kinase inhibitors [16,17], but the drug tolerance is the most common problem, seriously affecting the treatment effect [18]. There are many new treatment methods, such as gene therapy and bone marrow transplantation [19], but they cannot be applied on a large scale because of the difficult matching, high treatment costs and uncertain efficacy. Therefore, the selection of low-toxicity and high-efficiency drugs is a major clinical issue.

Traditional Chinese medicine has been used in the treatment of leukemia for a long time, and arsenic trioxide is mainly used to treat acute promyelocytic leukemia [20,21]. At present, some traditional Chinese medicine mixtures or monomers have been used in the treatment of CML and relevant studies argue that the therapeutic effect

of such drugs is similar to that of other therapy drugs, namely those promoting tumor cell apoptosis [22,23]. The mechanisms of action include blocking cell cycle, inducing cell necrosis, affecting apoptosis gene expression and inducing differentiation [24,25]. It was found in this study that puerarin could effectively inhibit the viability of K562 cells of CML. Furthermore, flow cytometry showed that puerarin could effectively increase the rate of apoptosis. The apoptosis-inducing effect was increased with prolonging the time of exposure in a dose-dependent manner. WB results showed that puerarin could induce the increased expression of pro-apoptotic proteins and decreased the expression of anti-apoptotic proteins in K562 cells, proving thus the effectiveness of puerarin in promoting K562 cell apoptosis.

Recently, with the increasing number of studies on autophagy, it has been found that a kind of autophagy can induce apoptosis [26,27], which mainly occurs in the process of tumor cell apoptosis, known as autophagic apoptosis [28,29]. In our study, the promoting effect of puerarin on K562 cell apoptosis was investigated. It was found that puerarin induced apoptosis while the autophagy level was increased, autophagy fluorescence was enhanced significantly, and autophagy-related protein expression was also increased, while autophagy was also dose-dependent. The results confirmed the induction of puerarin of K562 cell autophagy. In the grouping study of action of 100 μM puerarin for different times, it was noticed that the proportion of late apoptotic cells after 72 hrs was increased compared with that for 48 hrs, but there was no significant difference in the proportion of early apoptotic cells. The autophagy intensity in the 48 hrs group was slightly higher than that in the 72 hrs group, which may prove that puerarin-induced autophagy is associated with early apoptosis. Another reason is that autophagosomes have been dissolved by lysosomes in late apoptosis.

In order to clarify that autophagy is the main mechanism of pro-apoptotic effect of puerarin, autophagy inhibitor 3-MA was added into the puerarin-treated group. The experimental results showed that the inducing effect of puerarin on autophagy was significantly decreased, and the autophagy fluorescence and autophagy-related proteins were decreased after autophagy of K562 was blocked. At the same time, it was found that the inducing effect of puerarin on apoptosis was also significantly decreased, proving that the inducing effect of puerarin on apoptosis depends on the activation of autophagy and autophagic apoptosis plays an important role in puerarin-activated apoptosis.

Conclusion

Puerarin increases cell apoptosis through increasing the autophagic apoptosis of K562 cells, and autophagy inhibitors can inhibit the pro-apoptotic effect of puerarin.

Conflict of interests

The authors declare no conflict of interests.

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