

RESEARCH ARTICLE

Autophagy Inhibition Sensitizes Cisplatin Cytotoxicity in Human Gastric Cancer Cell Line Sgc7901

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Abstract

We aimed to investigate the mechanism and effects of autophagy on cisplatin (DDP)-induced apoptosis in human gastric cancer cell line SGC7901. After SGC7901 cells were treated with DDP and/or chloroquine, cell proliferation was measured using MTT assay; cell apoptosis was determined by flow cytometry; autophagy and apoptosis-related proteins expression were detected by Western blot; and quantitative analysis of autophagy after monodansylcadaverine (MDC) staining was performed using fluorescence microscopy. We found after treatment with 5 mg/L DDP for 24 h, the rates of cell apoptosis were $(21.07 \pm 2.12)\%$. Autophagy, characterized by an increase in the number of autophagic vesicles and the level of LC3-II protein was observed in cells treated with DDP. After inhibition of autophagy by chloroquine, the rates of cell apoptosis were increased to $(30.16 \pm 3.54)\%$, and the level of Caspase-3 and P53 protein were increased, and Bcl-2 protein was decreased. Therefore, autophagy protects human gastric cancer cell line SGC7901 against DDP-induced apoptosis, inhibition of autophagy can promote apoptosis, and combination therapy with DDP and chloroquine may be a promising therapeutic strategy for gastric cancer.

Keywords: Autophagy - cisplatin - chloroquine - apoptosis - gastric cancer

Asian Pac J Cancer Prev, **14** (8), 4685-4688

Introduction

Chemotherapy is the main method to cure gastric cancer. As the traditional Chemotherapeutic drug, cisplatin is used throughout all stage of chemotherapy on gastric cancer, such as ECF protocol of adjuvant chemotherapy, DCF protocol of palliative chemotherapy and PF+ Trastuzumab protocol of molecular targeted therapy, all of which are recommended in NCCN Guide as the I type proof (Cunningham et al., 2006; Van Cutsem et al., 2006; Bang et al., 2010). It's demonstrated in the study that the terminal gastric cancer patients cured by cisplatin protocol gain better therapy effects with longer lifetime (Pasini et al., 2011). It's deemed that the chemotherapeutic drugs kill the cancer cells mainly by the way of inducing cancer cells apoptosis. The autophagy is the process that intracellular proteins, lipids and organelles are separated and degraded in eucaryote, which maintains the balance of cellular metabolism and plays an important role in cancer development and therapy (Levine and Kroemer, 2008). It's reported lately that there is autophagy when cisplatin induces lung cancer cell A549 apoptosis, which will result in cancer cell survive finally (Ren et al., 2010). However, there is no domestic and abroad report on whether cisplatin induce the gastric cancer cell autophagy and the influence of autophagy on apoptosis. It's aimed in the study that the changes of gastric cancer cell SGC7901 are observed before and after autophagy, as well as the influence of

autophagy combined inhibitor - cisplatin on autophagy and its mechanism are preliminarily discussed.

Materials and Methods

Cell Culture

The human gastric cancer cell strain SGC7901 was purchased from Animal Experiment Center of Sun Yat-Sen University, inoculated on DMEM complete medium (produced by Gibco Company) containing 10% fetal calf serum, 100U/mL penicillin and 0.1mg/mL streptomycin then incubated in incubator under 37 °C, 5% CO₂ and saturation humidity.

Cell Grouping and Drug Treatment

There are 4 groups in the experiment as following: control group, chloroquine (CQ) group, cisplatin (DDP) group and combined group (CQ+DDP) (CQ and DDP are produced by American Sigma-Aldrich Company). The drug dosage of control group is zero; 20 μmol/L CQ was adopted in CQ group, and 5 mg/L DDP was adopted in DDP group. The combined group was treated by 20 μmol/L CQ for 1h, then added 5 mg/L DDP. The cells of each group were observed and detected after drug treated for 24 h.

Cell Viability Test by MTT Method

The cells in logarithmic phase are chosen to inoculated on 96-well plate. After incubated for 12 h, DDP and/or CQ with the fixed concentration were added. There are 9

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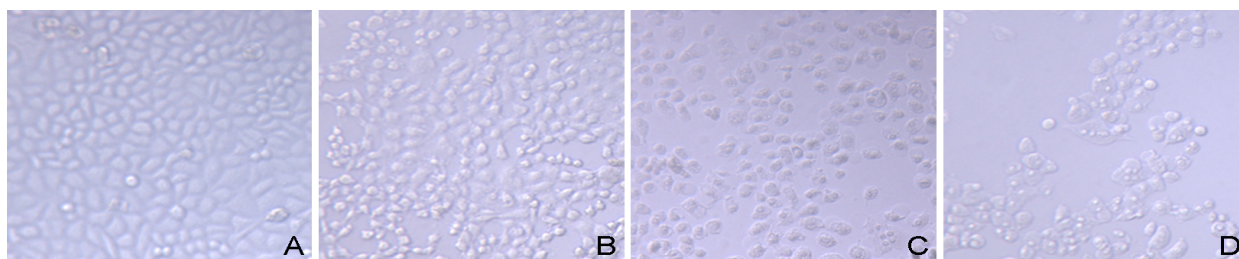


Figure 1. The Morphologic Change of SGC7901 Cells by Inverted Microscope ($\times 200$); A: Control; B: Chloroquine; C: DDP; D: Chloroquine+DDP

duplicate wells for each group with the volume of 200 μL in each well. After treated for a while, 20 μL MTT solution of 3 g/L was added into each well, then the cell strains were transferred to incubator for further incubating for 6h. The substrate was drawn and dropped. 150 μL DMSO was added into each well then vibrated and shaken. Enzyme mark instrument was adopted to detect the absorbance value under the wavelength of 490nm.

Apoptosis Test by AnnexinV-FITC/PI double staining flow cytometer

The cells in logarithmic phase are chosen to inoculate on 96-well plate. DDP and/or CQ with the fixed concentration were added. After 24 h, the cells of treated group and control group were collected and digested by no EDTA trypsinase. The total cells were collected and washed twice by PBS with supernatant fluid dropped. 400 μL binding buffer was added to re-suspend the cells. After added 6 μL AnnexinV-FITC and 4 μL PI, the cells were incubated for 15 min. The apoptosis was detected in red and green fluorescence channels with excitation wavelength of 488 nm.

MDC Staining and Apoptosis qualitative detection by fluorescence microscope

The cells in logarithmic phase are chosen to inoculate in culture flask. When there is anchorage growth with the stand density of 80%-90%, the original culture liquid was dropped, and the cells were washed with PBS solution. The cells of each group were treated correspondingly. The cells were cultured for 24 h under 5% CO_2 , 37°C and saturation humidity, then incubated for 60 min after adding 0.05 mmol/L MDC solution, washed for 4 times by PBS and observed as well as photographed by inverted fluorescence microscope with excitation wavelength of 380nm and emission wavelength of 525 nm.

Autophagy and apoptosis relative protein test by Western Blot

The cells of treated group and control group were collected and decomposed by 120 μL RIPA. The lysate was collected in EP tube and supersonic inspected. The lysate was then centrifugated for 30 min at 14000 r/min with the supernatant fluid kept. The protein quantitative detection was conducted by Bradford method. After sampling, the sample was boiled out for 5 min. After cooled down on ice, the sample was centrifugated for 30s at 10000 r/min. The supernatant fluid was drawn to SDS-PAGE (200V, 45 min), transferred film for 1h at 100V and sealing incubated for 1h on PVDF film containing 0.5% defatted

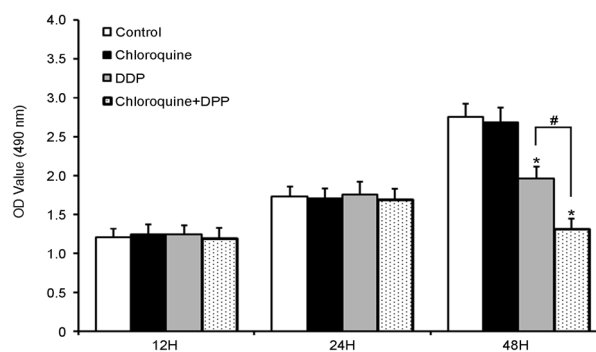


Figure 2. The Effect of DDP and Chloroquine on Cell Viability of SGC7901 Cells

milk at room temperature. It's incubated with the primary antibody overnight, and the film was washed twice by 0.1% (V/V) TBST. The secondary antibody incubation at room temperature was conducted for 1h, and then the film was washed for 3 times as well as conducted the coloration by ECL method.

Statistical Method

The experimental data are indicated as $\pm s$ and analyzed by the statistical software SPSS13.0. The measure materials between groups are compared by T-test. The result of the comparison between experimental group and control group is $P < 0.05$, which is statistical significant.

Results

The influence of cisplatin and chloroquine on the cell viability of SGC7901

After treated by drugs for 24 h (when the experiment process lasts for 48 h), there is no change of cellular morphology in CQ group (B), while there are obvious Cell shrinkage and anchorage separate in DDP group (C) and Combined Group (D) (Figure 1). Before treated by drugs, there is no significant difference on cell proliferation speed among all groups ($p > 0.05$). After treated by drugs for 24 h, there is no significant difference on cell proliferation speed between CQ group and control group, while the cell proliferation speed of DDP group and combined group is significantly lower than the control group with cell proliferation speed decreased. Moreover, the cell proliferation speed of combined group is significantly lower than DDP group (#) with $p < 0.01$ (Figure 2).

Cisplatin induce SGC7901 autophagy

There are plenty of acid autophagic vacuoles, and Monodansylcadaverin (MDC) can be absorbed by cell or selectively gathered in autophagic vacuole, which is

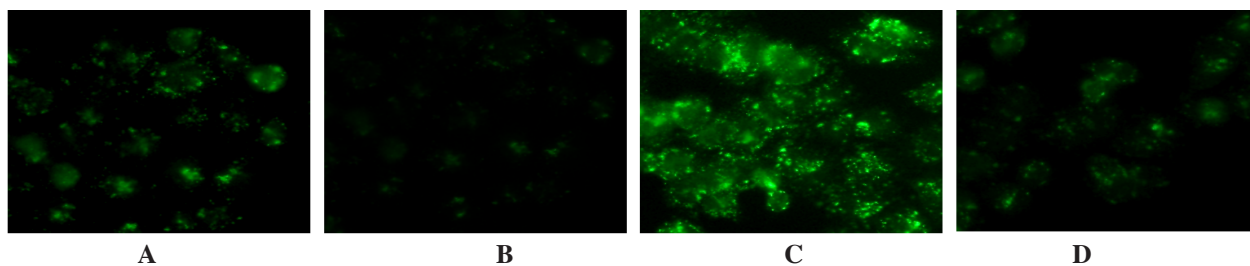


Figure 3. Autophagy Vesica Observed by Fluorescent Microscope (MDC staining $\times 400$); A: Control; B: Chloroquine; C: DDP; D: Chloroquine+DDP

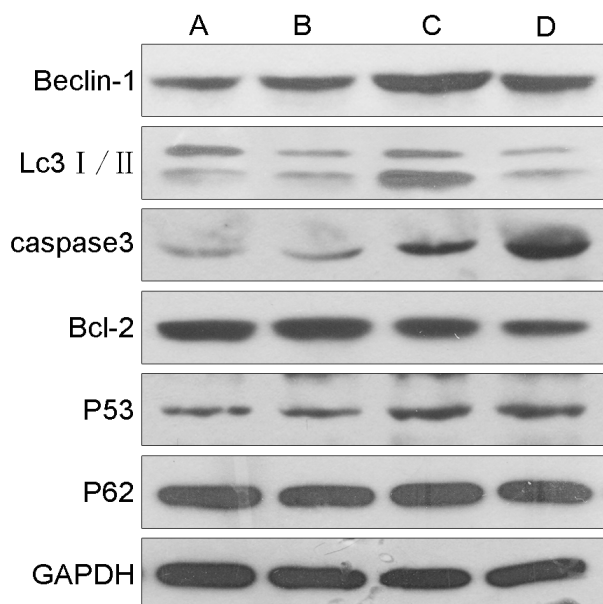


Figure 4. The Expression of Proteins of SGC7901 Cells by Western Blot; A: Control; B: Chloroquine; C: DDP; D: Chloroquine+DDP

treated by DDP at the fixed concentration for 24 h, the protein expression of Beclin-1 and LC3-II increase, and the ratio of LC3-II/Isignificantly increases ($p < 0.01$); After combined treated by CQ, the ratio of LC3-II/Ibegins to decrease (Figure 4). After treated by drugs for 24 h, the apoptosis relative protein expression of CQ group (B) is no significant different from the control group (A); the expression of Caspase-3 and P53 in combined group (D) enhances with a statistical significance compared with DDP group (C), while the expression of Bcl-2 declines with a statistical significance ($p < 0.05$). However, P62 expression has no obvious change (Figure 4).

Chloroquine promoting cisplatin to induce SGC7901 apoptosis

After the gastric cancer cell SGC7901 was treated by drugs for 24 h, the apoptosis rate of CQ group (B) is no significantly different from the control group (A) ($p > 0.05$), while the apoptosis rate of DDP group (C) and combined group (D) increases, and the apoptosis rate of combined group ($30.16\% \pm 3.54\%$) is significantly higher than the DDP group ($21.07\% \pm 2.12\%$) with $P < 0.05$ (Figure 5).

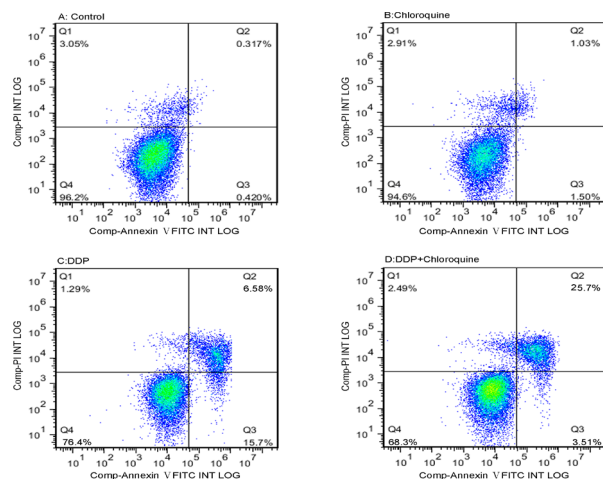


Figure 5. The Apoptosis Rate of SGC7901 Cells Detected by AnnexinV-FITC/PI Double-stained

one of typical morphological characteristics about the activated autophagy. After stained by MDC, It's observed under inverted fluorescence microscope that, as treated by drug for 24 h, the autophagic vacuole of DDP group (C) significantly increase compared with control group (A); after treated by CQ as a autophagy inhibitor, the autophagic vacuole obviously decrease (Figure 3, the spotted fluorescence structure around the nucleus is autophagic vacuole). It's shown in the Western blot result that, compared with control group, when SGC7901 was

Discussion

The incidence of gastric cancer in most regions of the world decreases recently. It's demonstrated by authoritative data that there are 738000 patients died from gastric cancer in 2008 in the world, which is ranked the second position about the cancer relative death. Chemotherapy is one of three therapy methods for gastric cancer. As a classic effective chemotherapy drug, cisplatin plays a critical role in postoperative adjuvant chemotherapy and palliative chemotherapy on gastric cancer (Cunningham et al., 2006; Van Cutsem et al., 2006; Bang et al., 2010). It's a pity that most patients finally fail to be treated by chemotherapy due to drug tolerance. The median survival period of terminal gastric cancer is ranged from 9.2 months to 13.8 months (Van Cutsem et al., 2006; Bang et al., 2010). It's manifested by the research that the combination of chemotherapy drug and autophagy modulator improve prognosis of cancer patients, reverse the drug tolerance. Therefore, cell autophagy has become the research hotspot on chemotherapy drug tolerance (Chen et al., 2010; Levy et al., 2011). Sotelo et al. (2006) conducted the random clinical experiment on II stage. It's demonstrated that, compared with placebo, the median survival period of glioblastoma postoperative patients who took oral chloroquine 150 mg per day for 12 months extended at a fixed degree based on the traditional

chemotherapy and radiotherapy.

Ashford TP found the autophagy phenomenon through electron microscope in 1962. The concept of autophagy was raised firstly by Christian de Duve who gained Nobel Prize in physiology in 1963 (Ashford, 1962; Klionsky 2008). Autophagy is a biochemistry process that cytoplasm and organelles are degraded by lysosome just like “self-digestion” in eukaryotic cell, which is also called as II type programmed cell death (Levine et al., 2004). During this process, cytoplasm and organelles are firstly wrapped by double layer membrane to form autophagic vacuole, merged with lysosome to form autophagic lysosome then, and finally degraded with wrapped. Autophagy has the dual function of protecting and killing cancer. However, most autophagy maintains the survival of cancer cells and keep them from apoptosis (Mathew et al., 2007).

It's reported by Ren et al., (2010) that, cisplatin induce lung cancer cell A549 apoptosis, meanwhile it leads to autophagy to result in the cancer cells survival. There is no domestic and abroad report on whether cisplatin induces the autophagy of gastric cancer cell SGC7901. It's observed in our study that, after gastric cancer cell SGC7901 treated by 5 mg/L cisplatin for 24 h, the cell proliferation vitality declines, apoptosis enhances, the number of autophagic vacuole increases, and Beclin-1 and LC3-II expression significantly raises. It's proven by all these results that cisplatin induces autophagy when it induces the apoptosis of gastric cancer cell SGC7901.

As a specific inhibitor for autophagy, chloroquine has the function of weakening the acid circumstance in lysosome, stabilizing the membrane of lysosome and enhancing the lethal effect of various chemotherapy drugs on cancer cells (Levy 2011; Kimura et al., 2013). In this study, compared with the control group, autophagic vacuole of CQ group is significantly less, and the rate of LC3-II/I is lower, but there is no significance on cell proliferation and apoptosis between two groups. It's pointed that 20 μ mol/L CQ can inhibit the autophagy viability of gastric cancer cell SGC7901, but cannot inhibit the cell growth by itself. As only treated by the chemotherapy drug of cisplatin, the autophagy of gastric cancer cell SGC7901 is induced. As combined treated by cisplatin and chloroquine, the autophagy viability declines significantly, and the apoptosis significantly enhances. It's illustrated by these results that the autophagy induced by cisplatin is protective autophagy, some of which antagonize the apoptosis of gastric cancer cell, and chloroquine has the function of promoting cisplatin to induce apoptosis by inhibiting autophagy viability. It's similar to the research result on gastric cancer cell treated by 3-MA and matrine which was studied by Li et al. (2013). It's also found that the expression of Caspase-3 and P53 is up-regulated in combined group, while the expression of apoptosis inhibiting protein Bcl-2 is down-regulated. It's pointed that the chloroquine induces gastric cancer cells switching on the programmed cell death and results in apoptosis dependent to Caspase by activating P53 and deactivating Bcl-2 after specific inhibiting autophagy. Accordingly, chloroquine enhances chemotherapy sensibility of cisplatin on gastric cancer cell SGC7901.

In brief, as the common drug for gastric cancer chemotherapy, cisplatin elicits the protective autophagy as inducing gastric cancer cell SGC7901 apoptosis. Cisplatin with the autophagy inhibitor chloroquine added strengthens the oncolytic effects on gastric cancer cells in order to increase the chemotherapy sensitivity. Thus, It's expected that chloroquine combined chemotherapy drugs will become the new therapy strategy for gastric cancer, which is worthy for further research on its internal effect and clinical therapeutic effectiveness.

Acknowledgements

This work was supported by the Jiangxi Province Youth Science Fund (No. 20122BAB215029).

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