Cisplatin-Induced Apoptosis is Promoted via Cisplatin-Induced Autophagy in Bladder Cancer Cells

Zhiqiang Fan, Xuejun Huangfu, Zhonghua Liu*

Department of Urology, Henan Provincial People's Hospital, Zhengzhou City, 450003, China

ABSTRACT

Objective: To investigate the effects of autophagy in cisplatin-induced apoptosis of bladder cancer cells. Methods: Bladder cancer cell T24 was regarded as cell model. The transmission electron microscope was used to detect autophagic vacuoles and the fluorescence microscope to detect the fluorescence accumulation profile of vectors for green fluorescent protein and microtubule associated protein 1 light chain 3 fusion protein (GFP-LC3). Protein immunoblotting was applied to detect the accumulation of LC3-II, thus detecting whether cisplatin could induce the bladder cancer T24 cell autophagy. Moreover, protein immunoblotting was also used to detect the autophagic relative signal pathway mammal target of rapamycin (mTOR) and the variation of ribosomal protein S6 kinase (P70S6K) with relative molecular mass 70 000 in downstream as well as the cleavage of apoptosis marker protein poly ADP-ribose polymerase (PARP). Afterwards, with the utilization of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), the viability changes in cisplatin-induced bladder cancer cells under the condition of autophagy promoting the presentation or absence of rapamycin were observed. Moreover, RNA interference was also adopted in this experiment to knockdown the LC3 expression. Results: Compared with the control group, the electron microscope revealed that cisplatin was able to induce plenty of autophagic vacuoles in bladder cancer cells. CFP-LC3 aggregation was viewed by the fluorescence microscope, showing a significant higher in cisplatin group than control group. The results of LC3 detected by protein immunoblotting indicated that the LC3-II content in cisplatin group was significantly enhanced with the prolongation of time and increase of cisplatin concentration. Especially, at the concentration of 50 and 100 μmol/L for 48 h with cisplatin treatment, the gray value of LC3-II/Actin (%) increased 30 and 44, respectively. Cisplatin treatment inhibited the phosphorylation of mTOR/P70S6K, and its phosphorylated strips were almost completely inhibited in the 100μmol/L for 48 h with cisplatin treatment. MTS results showed that cisplatin was able to lead to the loss of cell viability, which was 12% and 35% at the concentration of 50, and 100μmol/L for 24 h with cisplatin treatment. Moreover, the cell viability loss in autophagy-induced rapamycin and cisplatin combined treatment group was larger than that with single-use cisplatin treatment in control group (F = 74.890, P < 0.01). Besides, RNA interference experiment revealed that knocking down autophagic relative gene LC3 could reduce the PARP cleavage induced by cisplatin and the apoptosis was decreased. Conclusion: Cisplatin could induce autophagy in bladder cancer cell T24, which promoted cisplatin-induced apoptosis.

Keywords: Cisplatin; Bladder cancer; Autophagy; Apoptosis

Bladder cancer ranks a top morbidity in urinary system in China [1]. Bladder cancer invading lamina muscularis is especially a fatal disease, and the median survival time is merely 15 months in case of metastasis [2]. Cisplatin
(CP) is extensively applied platinum-based chemotherapeutics in clinic, which plays an important role in the chemotherapy of bladder cancer. Either in the classical methotrexate plus vincristine plus Adriamycin plus cisplatin (M-VAC) regimen or in the recently popular gemcitabine–cisplatin (GC) regimen, cisplatin reveals a crucial effect [3]. It is necessary to further learn the mechanism of cisplatin on bladder cancer cells. In recent years, researches on cell death have showed that programmed cell death, apart from apoptosis, also includes autophagic cell death and necrotizing apoptosis, which provides a new thought for tumor therapies [4].

Autophagy is referred to a basic life process to maintain steady status for which cells is highly conserved during evaluation, involving three major types: macroautophagy, microautophagy and chaperone-mediated autophagy. The autophagy in this study is mainly referred to macroautophagy [5]. Autophagy can degrade organelle and macromolecular protein. In normal circumstances, autophagy within cells is kept in a relatively low basic level, whereas when cells was affected by adverse factors, such as starvation and growth factor deficiency, autophagy level can be rapidly enhanced in order to maintain the supplies of energy and nutrient. Therefore, autophagy is generally regarded as the survival mechanism of cells, but the excessive activation of autophagy also can induce cell death. Autophagic cell death is also called programmed cell death II, while the relationship between autophagy and apoptosis is also very complicated, moreover, autophagy can inhibit, promote or accompany the apoptosis [6,7]. Similarly, researches in tumors also have showed that on the one hand, autophagy can be took as a protective factor for certain tumors to escape death, on the other hand, autophagy may inhibit tumors, for example, autophagic gene deficiency is able to induce tumor growth and the autophagic level is decreased after partial tumors have progressed. Moreover, researches also have been discovered that inhibition of autophagy can indirectly inhibit the drug-induced cell death [8-10]. Hence, autophagy also can become a research hotspot in the tumor drug therapy. The research in the human cervical cancer cell has revealed that inhibition of autophagy augment the cisplatin-induced apoptosis, which suggests that autophagy has a protective effect and may be one of the drug resistance mechanisms for tumors.

As to bladder cancer, the relationship between cisplatin and autophagy has not been reported at present. At the same time, it is still unclear that apart from inducing apoptosis, whether cisplatin has other programmed death methods or not. This study is to definitely ascertain whether there exists autophagy participating in the apoptosis process of cisplatin-induced bladder cancer cells and to investigate the impact of autophagy on cancer cell death.

**MATERIALS AND METHODS**

**Drugs and reagents**

McCoy'5A culture medium and penicillin-streptomycin were purchased from Nanjing KeyGEM Biotech. CO., LTD.; DMEM culture medium was purchased from America Hy-Clone CO., LTD; Fetal bovine serum was purchased from America GIBCO Company; Cisplatin, rapamycin and microtubule associated protein 1 light chain 3 (LC3) antibody were purchased from America Sigma-Aldrich Co. LLC; Z-VAF-FMK (Z-V-FMK) was purchased from R&D systems America Co., Ltd; Poly ADP-ribose polymerase (PARP), mammal target of rapamycin (mTOR) and relative molecular mass 70 000 ribosomal protein S6 kinase (P70S6K) antibody were purchased from America Cell Signaling Technology, Inc; Actin antibody, LC3 specificity siRNA and control siRNA were purchased from America Santa Cruz Biotechnology, Inc.
Instruments
CO₂ cell incubator was purchased from America NUaire Company; Benchtop centrifuge was purchased from America Thermo Inc.; Benchtop high speed refrigerated centrifuge was purchased from America Sigma-Aldrich Co. LLC; -20ºC low-temperature refrigeration was purchased from Haier Electronics Group Co., Ltd (China); -80ºC low-temperature refrigeration was purchased from Heraeus Holding GmbH (German); AL204 electronic scale was purchased from America Mettler-Toledo International Inc.; (Axio Imager (A1) was purchased from Carl Zeiss Group Co., Ltd (German); Mini Trans-Blot electric whirl apparatus was purchased from Bio-Rad Laboratoried, Inc. (America); Electrophoresis tank AE-6500 type was purchased from ATTO Corporation (Japan); JY600C electrophoresis apparatus under constant voltage and constant current was purchased from Beijing Junyi East Electrophoresis Equipment, Ltd. (China); MilliQ plus ultrapure water systems was purchased from Merck Millipore Corporation (America).

Cell culture and siRNA interference
Human bladder cancer cell line T24 was purchased from ATCC Corporation (America) and preserved in the Xuejun Jing laboratory of Institute of Microbiology Chinese Academy of Science. Cells were cultured with McCoy’5A culture medium containing 10% (volume fraction) fetal bovine serum. Upon the concentration of cell growth was close to 100%, the previous culture medium was discarded, being washed once with PBS. Pancreatin (without EDTA) was used to digest the adherent cells. After 1 500 r/min centrifugation in 2 min, the cells were harvested. Meanwhile, the supernatant was discarded and fresh culture medium was added for suspension. Cells were assigned into the new culture dish and kept overnight in order to achieve 60%-70% density for cell growth. At the same time, the culture medium was replaced with fresh one. Cisplatin with the final concentration with 50 μmol/L and 100 μmol/L was respectively added for stimulation. Then, after 6, 12, 24 and 48 h, the cells were harvested.

Before 12 h of the preparation of siRNA interference experiment, the needed cells were incubated into culture dish in 30% density in accordance of the above mentioned methods. Referred to the instruction of transfection reagents, the siRNA and transfection reagents were added into DMEM culture medium without serum, respectively. Mixing those after 5 minutes’ standing and standing 20 minutes again, the mixed DMEM culture medium was added into the cell culture dish which had been replaced with new culture medium (maintaining blood serum). With interference 36 h, it was changed with fresh culture medium. Further, Cisplatin with 50 μmol/L concentrial was added for stimulation 24 h. Finally, the cells were collected.

Preparation of specimens of electron microscope
T24 cells were incubated in the culture dish with 6 cm diameter and cultured overnight in the 5% (volume fraction) CO₂ cell incubator at 37ºC until the cell density reached 60%-70%. Then changing fresh culture medium, ciaplatin with 50 μmol/L final concentration was added for 6 h treatment. After the treatment time was achieved, the pancreatin (without EDTA) was immediately used to digest cells. After 1000 × g centrifugation in 5 min at 4ºC, the cells were harvested, being washed twice with cold PBS, added with 3% (volume fraction) glutaraldehyde solution, fixed at 4ºC and finally sent for examination.

Plasmid transfection and fluorescence microscope observation
In the 12 h before transfection, the needed cells were incubated into culture dish in 80% density in accordance of the above mentioned
methods. Referred to the instruction of transfection reagents, the green fluorescent protein and microtubule associated protein 1 light chain 3 fusion protein (GFP-LC3) plasmid and Lipofectamine 2000 transfection reagents were added into DMEM culture medium without serum, respectively. Mixing those after 5 minutes’ standing and standing 20 minutes again, the mixed DMEM culture medium was added into the cell culture dish which had been replaced with new culture medium (maintaining blood serum). With 36 h transfection, the cells were incubated into the 6-well plate with cover slip for 12 h culture. After cisplatin with 50 μmol/L final concentration was added in 6 h treatment, the previous culture medium was discarded. Moreover, fixed in 15 min with 40 g/L paraformaldehyde solution at room temperature and washed twice with PBS, the cells were finally performed DAPI staining and treated with mounting. The made-up cell slides were used to observe the GFP-LC3 fluorescence accumulation profile with the utilization of fluorescence microscope.

**Cell collections and protein immunoblotting**

Cells were treated in appointed time, with culture solution discarded and cold PBS washed once. Then, cell lysis buffer was added in a certain amount, thus making up the followings: 1 mL mother liquid [1% (volume fraction) Triton X-100, 10% (volume fraction) glycerinum, 50 mmol/L Hepes, pH 7.4], 20μL 5 mol/L NaCl, 10 μL 0.5 mol /L EGTA/EDTA, 10 μL 0.1 mol /L NaF, 20 μL 0.1 mol /L PMSF, 2 μL DTT, 2μL Na3VO4, 1 μL Protease inhibitor. With the utilization of cell spatula, all cells in the culture dish were collected equably. The collected cell lysis buffer was placed on ice for 20 min pyrolysis. After 13 000 r/min centrifugation in 15 min, the supernatant was collected, with which the loading buffer was added and be boiled. According to needed protein relative molecular weight, 80 g/L (separating Actin and LC3) polyacrylamide gel was prepared. At the same time, loading glue leaking was applied to separate protein and then the protein was transferred into PVPF membrane. Afterwards, the PVDF membrane with transferred protein was treated according to the following procedure: confining liquid was used for sealing film 60 min, with incubation overnight for primary antibody and 90 min for antibody respectively. After appropriate rinsing, the fluorescence solution was smeared and exposure developing in darkroom was conducted. Finally, the negative films were scanned and analyzed.

**Detection on cell survival rate by 3-(4,5-dimethylthiazol-2-yl) -5-( 3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt**

The prepared T24 cells was digested and incubated in 96-well plate equably. After incubation overnight, it was replaced with DMEM culture medium without phenol red. Each 3-well was regarded was a parallel control group. Cisplatin was added with the final concentration of 25 μmol/L, 50 μmol/L and 100 μmol/L and that of 0 μmol/L in blank control group. Each concentration was divided into two groups, with one added with ciaplatin and rapamycin in 10 nmol/L final concentration and the other added cisplatin and DMSO in equivalent solvent as control. After treated with 24 h, every well was added with 20μL mixed solution of MTS and PMS (20:1). Enzyme linked immunosorbent detector was adopted to detect optical density value of various wells at 490 nm wavelength, and the results were recorded.

**Statistical analysis**

MTS experiment results were analyzed in pairwise comparison with one-way analysis of variance and Student-Newman-Keuls (S-N-K). SPSS 13.0 software was used for treatment. α = 0.05 was regarded as inspection level.
**RESULTS**

*Cisplatin-induced autophagy and apoptosis of bladder cancer cells*

Transmission electron microscope was one of the most reliable approaches to observe autophagy, and also was the autophagy method which was first discovered and reported [11,12]. With the utilization of electron microscope to observe T24 cells (Figure 1), it was found that comparing control group (Figure 1A), the cisplatin treatment group (Figure 1B) formed a large number of autophagosome. GFP-LC3 was an autophagic marker protein together with green fluorescence. During cells was in a lower autophagic level, GFP-LC3 was dispersively distributing in cells, most in cell nucleus. Whereas when the autophagic level rose, the autophagosome augmented and GFP-LC3 was combined with autophagosome in an esterification formation, representing a fluorescence distribution of punctiform aggregation [13]. The results of fluorescence microscope indicated that with cisplatin treatment, the fluorescence aggregation degree of T24 cells was significantly risen (Figure 1C and D).

LC3 was an autophagic marker protein, including LC3-I and LC3-II subtypes. The expression quantity of LC3-II showed a direct proportion with the degree of autophagy (13). Hence, with the assistance of immunoblotting, LC3 and internal reference Actin were detected. We found that as the treatment time prolonged, especially on 24 h and 48 h, the value of LC3-II/Actin appeared a significant increase. Meanwhile, when the increment of the higher concentration (100μmol/L) was larger than that of lower concentration (50μmol/L). Especially on 48 h, the gray value of LC3-II/Actin (%) under the treatment of 50 and 100μmol/L cisplatin were enhanced 30 and 40, respectively (Figure 1E).

mTOR pathway was the generally conceived autophagic negative regulation pathway (14). Thus we took a detection and found that similarly, in the situation of longer time (24 h and 48 h) and higher concentration (100μmol/L), cisplatin treatment significantly inhibited the phosphorylation of mTOR and downstream P70S6K (Figure 2). At the same time, the apoptotic marker protein PARP which was the cleavage substrate of caspase was detected (15). PARP cleavage was the marker of the occurrence of apoptosis. After the cisplatin treatment, we detected the cleavage of PARP, which was confirmed the occurrence of apoptosis. However, the appeared time (24 h) of cleavage strips was posterior to that of LC3-II, and its expression quantity showed time and dose dependency (Figure 2).

*Cisplatin-induced apoptosis of bladder cancer cell was promoted by autophagy*

1. Killing effect of cisplatin on T24 cells was augmented with autophagy-promoted rapamycin

Rapamycin was able to specifically inhibit mTOR, an universally adopted autophagic accelerant (16). To verify the effect of autophagy on cisplatin-killed tumor cells, on the treatment condition of cisplatin and rapamycin in co-existence, the cell viability was detected by applying MTS method. The results showed that on the 24h' treatment with 50 μmol/L and 100 μmol/L, the cell viability was decreased by 12% and 35%, respectively. Besides, the cell viability loss in autophagy-promoted rapamycin and cisplatin combined treatment group was larger than that in control group with single-use cisplatin treatment. Through variance analysis, differences among groups showed a statistical significance (F=74.890, P<0.01). S-N-K pairwise comparison indicated that comparisons between every two groups with 25μmol/L, 50μmol/L and 100μmol/L cisplatin concentration also showed a statistically significant difference (Figure 3A).
Fig. 1 Cisplatin induced autophagy in T24 cells (Note: T24 cells were viewed by electron microscope after treated with blank control and cisplatin (50 μmol /L) for 6 h and collected as described above in figure 1. Compared to control (A), autophagosomes (double-membrane structures which may have content in them) formation was obviously enhanced in cisplatin-treated cells (B). T24 cells were transfected with GFP-LC3 plasmid in C and D. C was control and cells were treated with cisplatin (50 μmol /L) for 6 h in D. In D, cisplatin enhanced GFP-LC3 aggregation and more positive dots were visualized than in C. E showed cisplatin induced LC3-II accumulation in T24 cells. T24 cells were treated with cisplatin (50 and 100 μmol /L) for 6, 12, 24 and 48 h and harvested as described in methods. LC3 and actin were analyzed by immunoblotting with specific antibodies. The gray value represented the amount of protein, and the ratios of LC3-II to actin are presented below the blots.)

Fig. 2 Cisplatin induced PARP cleavage and inhibited the phosphorylation of mTOR/P70S6K in T24 cells (Note: T24 cells were treated with cisplatin (50 and 100 μmol /L) for 6, 12, 24 and 48 h and harvested as described in methods. PARP, mTOR, P70S6K and actin were analyzed by immunoblotting with specific antibodies. The gray value represented the amount of protein. p: phosphorylation; t: total)

Fig. 3 Autophagy regulated cisplatin-induced cell viability loss and PARP cleavage (Note: A, MTS assay was carried out when cisplatin treated and rapamycin combined with cisplatin (25, 50, 100 μmol /L) for 24 h. Data are representatives of three independent experiments and presented as mean ± SD. Differences between groups were tested by one-way ANOVA and the result has statistical significance (F = 74.890, P < 0.01). Differences between two different groups were tested by S-N-K. * P < 0.05, indicates a significant difference between the two groups; B, After knocking down of LC3 (siLC3), T24 cells were treated with cisplatin (50 μmol /L) for 24 h. Then, cells were harvested and indicated proteins were analyzed by immunoblotting. Compared to mock (control siRNA), cisplatin-induced PARP cleavage was obviously reduced. c, cleavage.)
2 Knocking down autophagic relative gene LC3 expression to inhibit cisplatin-induced apoptosis

In order to further verify the above mentioned results, autophagic relative gene LC3 was interfered and took with cisplatin treatment. Compared with control group, after knocking down LC3, cisplatin-induced PARP cleavage was significantly reduced (Figure 3B).

DISCUSSION

Cisplatin is the representative drug of platinum as well as a non-cell periodic cytotoxic chemotherapeutics [17], having a crucial effect in the chemotherapy of bladder cancer [3]. Literature has indicated that cisplatin can induce bladder cancer cells appearing apoptosis and the occurrence of apoptosis is the main method in which cisplatin causes the death of bladder cancer cells [18]. Autophagy is not only a method which cells degrade their own organelle, but also a formation of cell programmed death. What’s more, more and more studies indicated that there existed an internal relationship between autophagy and apoptosis [19]. However, the relationship between autophagy and apoptosis has never been referred in the relative studies of bladder cancer. Through the utilization of electron microscope and immunofluorescent assay, this study was confirmed that cisplatin was able to induce bladder cancer cell T24 appearing cell autophagy, which appeared before apoptosis.

In the other tissues’ and cells’ studies, cisplatin-induced cell autophagic functions are different. For instance, the mice experiment has indicated that compared with control group, for mice with autophagic deficiency, cisplatin-induced acute kidney injury is more seriously and accompanied with adverse DNA injury and more apoptosis [20]. In the lung carcinoma cell A549 of cisplatin resistance, autophagic inhibitor 3-methyladenine (3-MA) inhibiting autophagy is able to strengthen the growth inhibition and apoptosis-promoting effect of cisplatin on cancer cells [21]. Moreover, the same results are also discovered in esophageal cancer cell EC9706 [22]; Using 3-MA in laryngeal carcinoma cell Hep-2 or knocking down Beclin-1 to inhibit autophagy can strengthen cisplatin-induced apoptosis [23]; In hepatoma carcinoma cell Huh-7 and HepG2, cisplatin is able to activate ATG7 to strengthen autophagy through down regulating miR-199a-5p and to induce cancer cell drug resistance [24]; Through activating adenosine monophosphate activated protein kinase (AMPK), human glioma cell U251 and mouse glioma cell C6 are able to inhibit mTOR up regulating autophagy to resist cisplatin-induced apoptosis [25]. However, the effects of autophagy in tumor therapies are very complicated, showing possibly different effects and mechanisms in different drugs and tumors [26]. Hence learning autophagy’s situation is very critical when cisplatin acts on bladder cancer cells. In this study, autophagy strengthening or inhibiting profile was designed in order to observe the effects of cisplatin on bladder cancer cells. Unexpectedly, the results showed that on the situation of autophagy promoting rapamycin, the killing effect of cisplatin was strengthened. Furthermore, during knocking down autophagic relative gene LC3, the PARP cleavage was reduced, suggesting that autophagy could promote cell death with cisplatin treatment on bladder cancer.

Studies have showed that cisplatin-induced apoptosis of bladder cancer cells is mainly through the signal pathway caspase depended and the regulation of p53, Bax, Bcl-2 and other proteins [18]. Studies have discovered that autophagy is possibly the upstream of apoptosis inducing or promoting apoptosis or a vital alternative pathway for autophagy, directly inducing cell death [27]. In this study, it was found that autophagy appeared before the occurrence of apoptosis. Interfering autophagy
was able to affect the apoptosis process, but the dual character of autophagy induced a certain problems in the tumor therapy. Hence, it was a must to understand the regulatory mechanisms of autophagy and tumor cell death. At present, the regulatory mechanisms researched in hot are mainly involving in the followings: cancer gene Bcl-2 abnormally expressed is able to inhibit autophagy or apoptosis, or inhibit the two simultaneously; Tumor relative signal pathways such as Erk, Akt and PI3L (I type) are able to promote cancer cell survival and inhibit autophagy by various approaches; Cancer suppressor factors such as TSC1/TSC2, PTEN and p53 are able to activate autophagy. Besides, cancer suppressor factors such as DAPk, Beclin-1, p19ARF and UVRAG are also directly participating the regulation of autophagy [27]. Moreover, studies also have found that autophagy is able to degrade activated caspase-8 [28], indicating a possible feedback regulation mechanism between autophagy and apoptosis. Although there are numerous study evidences, the complete regulatory mechanism is still unclear now. Therefore, the nodal point and signal pathway which autophagy acts on apoptosis will be further searched for in the future.

All in all, autophagy could be used as a new target spot to design new drugs or combined therapeutic regimens [29,30]. The results in this study suggested that in the treatment of bladder cancer, drugs to induce autophagy had the application prospect. Therefore, combining autophagic regulatory drugs and chemotherapeutics probably would become a new approach to treat bladder cancer.

REFERENCE


