Blockage of cisplatin-induced autophagy sensitizes cervical cancer cells to cisplatin

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ABSTRACT. Development of chemoresistance is a major obstacle that leads to the recurrence and progression of cervical cancer (CC). Autophagy, meaning, "eating of self", has shown paradoxical functions in tumors. In this study, we first investigated the process of autophagy induction by cisplatin in CC cells. Next, we investigated the role of autophagy in cisplatin-sensitivity of CC cells via blockage of cisplatin-induced autophagy. The results demonstrated that cisplatin induces autophagy in CC HeLa cells via upregulating the formation of autophagic vesicle, promoting the conversion of LC-I to LC-II, and increasing the expression of autophagy-related gene 7 (Atg-7). On the other hand, the autophagy inhibitor, 3MA, downregulated cisplatin-induced formation of autophagic vesicles, reduced the conversion of LC-I to LC-II, and decreased Atg-7 expression. Moreover, 3MA reversed the reduction in cellular viability and induction of apoptosis by cisplatin in HeLa cells. Our results imply that autophagy blockage may play a key role in the chemosensitivity of cervical cancers.

Key words: Autophagy; Chemosensitivity; Cervical cancer; Cisplatin

INTRODUCTION

Cervical cancer is the third most common cancer among women worldwide, with the fourth highest fatality rates among females (Jemal et al., 2011). In particular, it is still a severe health issue in developing countries, although the incidence of this disease is slowly decreasing in developed countries due to early diagnosis. The Asia Oceania region accounts for more than 50% of all cases and cervical cancer-related deaths worldwide (Ferlay et al., 2015). Radical treatment of cervical cancer still relies on surgical resection and pelvic node dissection (Verleye et al., 2009). Chemotherapy is one of the most useful strategies in systematic treatment of cervical cancer (Pearcey et al., 2007). Cisplatin-based concurrent chemoradiotherapy (CCRT) is a standard treatment for locally advanced cervical cancer (Lukka et al., 2002; Lanciano et al., 2005; Vale et al., 2008). However, development of chemoresistance poses a major impediment that contributes to tumor recurrence, progression, and certain deaths (Burger et al., 2011).

Autophagy means, "eating of self" and constitutes a basic cellular process of both physiological and pathological importance (Jaeger and Wyss-Coray, 2009; Gibbings et al., 2012). Studies have shown paradoxical functions of autophagy in tumors (Mathew et al., 2007; White and DiPaola, 2009). Autophagy promotes tumor growth during tumor development, and conspires with inflammation (Korkaya and Wicha, 2011; Chen and Guan, 2013). On the other hand, autophagy is also associated with tumor cell death. Tumor-promoting and -suppressive roles of autophagy have been found in the same mouse model of lung cancer (Chen and Guan, 2013). Moreover, autophagy has been shown to be a cell death promoter, and is involved in tumor suppressor mechanisms (Gozuacik and Kimchi, 2004). Therefore, it is not clear whether autophagy induced by chemotherapy contributes to tumor cell death or leads to chemoresistance.

In this study, we investigated autophagy induction by cisplatin in cervical cancer cells, and explored the role of autophagy in cervical cancer cells. Our results imply that autophagy blockage has a chemosensitivity effect on cervical cancers.

MATERIAL AND METHODS

Cell culture and treatment

Human cervical cancer HeLa cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA,) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), with 50 µg/mL penicillin and streptomycin (CSPC Pharmaceutical Group Limited, Shijiazhuang, China). Cells were incubated in a humidified chamber at 37°C in 5% CO_2. For autophagy induction, HeLa cells (>85% confluent) were treated with either 100 nM rapamycin (Sigma-Aldrich, St. Louis, MO, USA), 0.2 µg/mL cisplatin (Sigma-Aldrich, St. Louis, MO, USA) or both agents for 12, 24 or 48 h. For autophagy inhibition, HeLa cells were treated with 1 mM 3-methyladenine (3MA) for 12, 24, or 48 h.

Quantitative GFP-LC3 and electron microscopy analysis

The coding sequences of GFP and LC3 were cloned into pcDAN3.1(+) plasmids (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Quantitative analysis of acid vesicular organelles (AVO) formation was carried out on HeLa cells transfected with the GFP-LC3 plasmid. After transfection for 6 h, cells were subject to rapamycin or cisplatin treatment for

another 24 h. Images of GFP-positive vesicles were visualized via fluorescence microscopy.

Observation of autophagic vesicles under electron microscopy was performed as described previously (Mizushima et al., 2008). In brief, HeLa cells post-treatments were collected via centrifugation for 5 min at 4°C and 800 rpm. Isolated cells were then serially fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Cells were then incubated with 1% OsO_4 in cacodylate buffer for 1 h at room temperature. Post-dehydration with ethanol, cell pellets were embedded in Spurr resin for sectioning. Images of autophagic vesicles were observed under a transmission electron microscope (JEM-1230, Jeol, Tokyo, Japan).

Western blot analysis

Cellular proteins were extracted from HeLa cells post-treatment with a cell lysis reagent (Promega, Madison, WI, USA), and were quantified using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) according to the manufacturers protocol. Protein samples were first separated with 10% gradient SDS-PAGE gel, and were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with primary rabbit polyclonal antibodies against LC3 (Abcam, Cambridge, UK), autoghagy-related gene (Atg 7; Santa Cruz, CA, USA) or β-actin (Sinobio, Beijing, China), and was then probed with Goat anti-rabbit IgG (Pierce, Rockford, IL, USA). Finally, specific blots were visualized by enhanced chemiluminescence detection systems (Amersham Pharmacia Biotech, Amersham, UK). The blots were quantified by comparison of gray value of LC3 II to LC3 I, or by comparing the gray value of Atg7 to β-actin.

MTT assay and apoptosis analysis

Cell viability was determined by MTT assays. HeLa cells were seeded in 96-well plates, and allowed to attach overnight. Afterwards, cells were treated with 1 mM 3MA or (and) with 0.2 µg/mL cisplatin. After 12, 24, 36, or 48 h of incubation, cell viability was measured. The MTT assay was conducted according to the standard protocol. Absorbance was measured at 570 nm with a reference wavelength of 750 nm using a spectrophotometer.

Apoptosis of treated HeLa cells was analyzed with an annexin V-FITC apoptosis detection kit (Abcam, Cambridge, UK). HeLa cells, post-treatment with 1 mM 3MA or (and) with 0.2 µg/mL cisplatin for 24 or 48 h, were collected and suspended in ice-cold 70% ethanol overnight for fixation. HeLa cells were then stained with propidium iodide or annexin V-FITC after centrifugation and resuspensions. Apoptotic cells were assayed according to manufacturer instructions using a flow cytometer (BD FACScan). Results were expressed as the percentage of apoptotic cells from the total cells.

Statistical analysis

All statistical evaluations are reported as means ± SE. Data were analyzed by the Student t-test. The criterion for statistical significance was considered as $P < 0.05$.

RESULTS

Cisplatin induces autophagy in cervical cancer cells

Autophagy is characterized by the formation of autophagic vesicles, i.e., AVOs in the cy-

tosol (Paglin et al., 2001). Cytosolic AVOs can be observed under fluorescence microscope with GFP-LC3 labeled autophagosomes. To confirm autophagy induction in cervical cancer HeLa cells, we examined AVOs formation in HeLa cells, which were subject to 0.2 µg/mL cisplatin. As shown in Figure 1, compared to the control group (Figure 1A), there was significantly higher level of green fluorescent protein (GFP)-positive vesicles in the HeLa cells treated with 100 nM rapamycin (autophagy inducer, as positive control) ($P < 0.001$; Figure 1B) or with 0.2 µg/mL cisplatin ($P < 0.01$; Figure 1C-D).
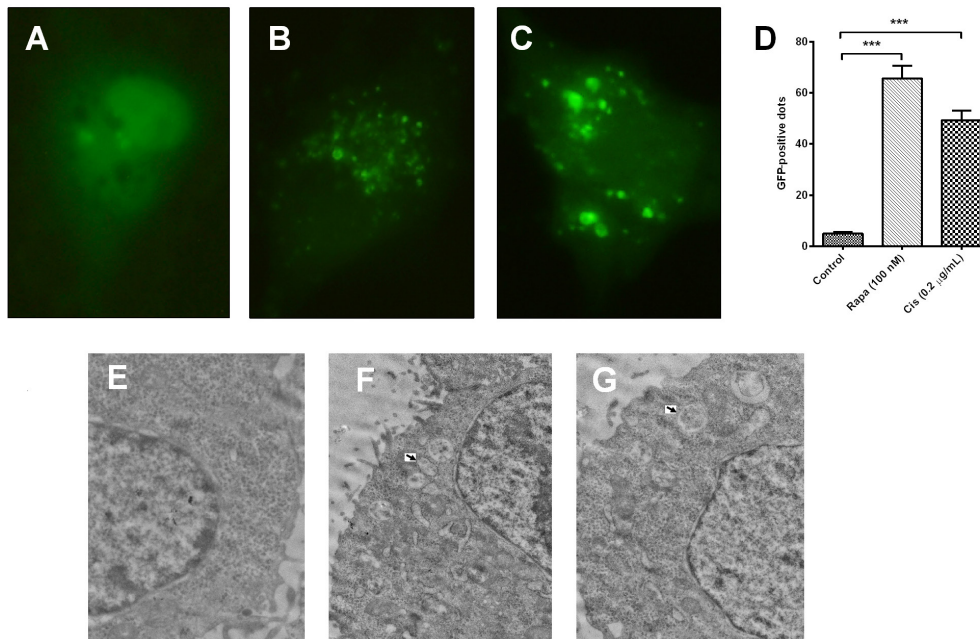
Figure 1. Cisplatin promotes autophagic vesicle formation in HeLa cells (A-C). Autophagic vesicle formation in negative control HeLa cells (**A**), in HeLa cells treated with 100 nM (**B**), or 0.2 µg/mL cisplatin (**C**). Autophagic vesicles are shown as green fluorescence protein (GFP)-positive dots (**D**). Number of autophagic vesicle HeLa cells after various treatments (E-G). Autophagic vesicles under electron microscopy in control HeLa cells (**E**), in HeLa cells treated with 100 nM (**F**), or with 0.2 µg/mL cisplatin (**G**). Autophagic vesicles are indicated by arrows. ***$P < 0.001$.

To further confirm autophagy induction by cisplatin in HeLa cells, we observed autophagosome formation via electron microscopy. As shown in Figure 1E-G, compared to the control HeLa cells (Figure 1E), in which autophagic vacuoles were rarely seen, there were numerous membrane vacuoles in the rapamycin-treated (Figure 1F) or the cisplatin-treated HeLa cells (Figure 1G).

Aside from the formation of AVOs, a high conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3) -I to LC3-II also occurs in autophagy, both of which play important role in autophagosome formation (Daido et al., 2004). To reconfirm autophagy induction by cisplatin, we then investigated the conversion of LC-I to LC-II via western blotting. As shown in Figure 2A and 2B, there was significant higher level of LC3-II compared to LC3-I in HeLa cells treated with rapamycin or cisplatin ($P < 0.001$). In addition, as demonstrated in Figure 2A and 2C, the level of the autophagy-related protein 7 (Atg-7) was also significantly upregulated by rapamycin and cisplatin in HeLa cells. Taken together, our results confirmed autophagy induction by cisplatin treatment in cervical cancer HeLa cells.
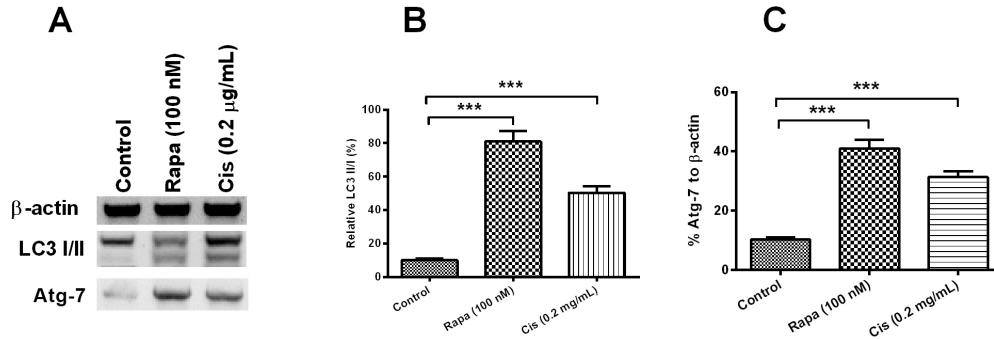
Figure 2. Cisplatin promotes autophagy-associated molecules in HeLa cells. **A.-C.** Western blot analysis of the conversion of LC3-I to LC3-II, and the autophagy-associated gene 7 (Atg-7) in control HeLa cells, and in HeLa cells treated with 100 nM or 0.2 µg/mL cisplatin. Statistical significance are shown as ***$P < 0.001$. Results are obtained from three independently performed experiments.

3MA reduces autophagy induction by cisplatin and sensitizes HeLa cells to cisplatin

To further determine the involvement of autophagy in the chemo-sensitivity of cervical cancer cells, we investigated induction of autophagy by cisplatin in HeLa cells with a chemical autophagy inhibitor, 3MA. As indicated in Figure 3A, formation of AVOs was significantly promoted in HeLa cells with singular cisplatin (0.2 µg/mL) treatment, whereas such promotion was reduced by 3MA treatment ($P < 0.01$; Figure 3A). Furthermore, the conversion of LC-I to LC-II as well as the level of Atg-7 were also significantly reduced by 3MA treatment ($P < 0.05$; $P < 0.01$; Figure 3B-D). Therefore, the autophagy inhibitor 3MA effectively reduced cisplatin-induced autophagy in cervical cancer HeLa cells.
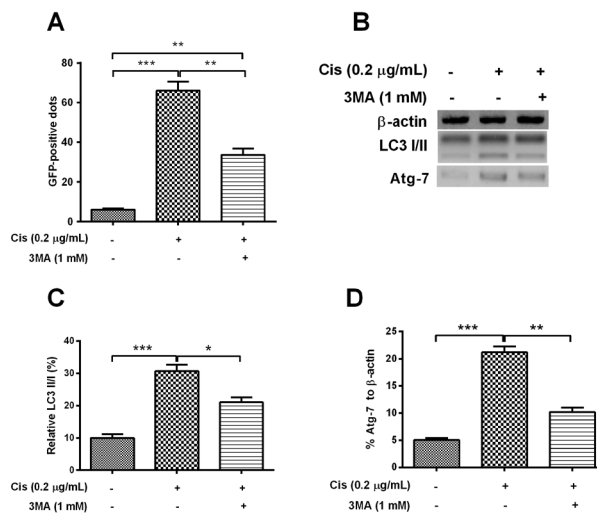
Figure 3. 3MA inhibits cisplatin-mediated promotion of autophagy-associated molecules in HeLa cells. **A.** 3MA reduced the formation of autophagic vesicles in HeLa cells treated with 0.2 µg/mL cisplatin; **B.** western blot analysis of LC3-I to LC3-II conversion and Atg-7 expression in HeLa cells treated with 0.2 µg/mL cisplatin or (and) 1 mM 3MA; **C.** relative levels of LC3-II to LC3-I in each group; **D.** percent levels of Atg-7 to β-actin in each group. All experiments were independently performed at least three times (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

To determine the influence of the inhibition of autophagy by 3MA on the sensitivity of HeLa cells to cisplatin, we examined cell viability post-treatments with 3MA, cisplatin, or both agents. Figure 4A demonstrated that cisplatin treatment significantly reduced the viability of HeLa cells, compared to the 3MA ($P < 0.01$ at 24 or 48 h post-treatment), as shown by the MTT assay. Moreover, the combined treatment significantly reduced viability of HeLa cells, as compared to singular treatments ($P < 0.001$ at 24 or 48 h post-treatment). In addition, the combined treatment with both cisplatin and 3MA significantly upregulated apoptosis in HeLa cells, as compared to with 3MA or cisplatin alone ($P < 0.05$ at 24 or 48 h post treatment). These results reveals that blocked autophagy by 3MA sensitizes cervical cancer cells to cisplatin treatments.
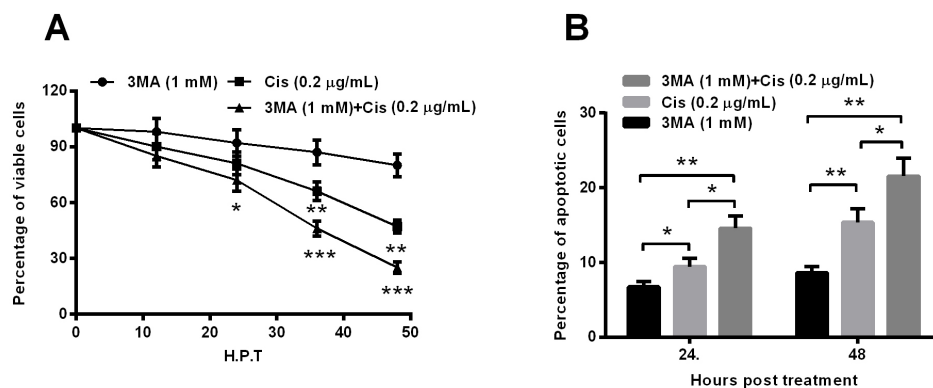
Figure 4. 3MA reduces cisplatin-mediated cell viability reduction and apoptosis induction in HeLa cells. **A.** Viability of HeLa cells post-treatment with 0.2 μg/mL cisplatin or (and) with 1 mM 3MA for 12, 24, 36 or 48 h; **B.** apoptosis induction by 0.2 μg/mL cisplatin or (and) with 1 mM 3MA for 24 or 48 h. Each result was averaged from three independent experiments. *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$.

DISCUSSION

Chemoresistance has been shown to be the main obstacle for radical treatments of cervical cancer and other types of malignances (Benedetto et al., 2015). Accumulating studies have indicated that autophagy plays an important role in the chemo-resistance of cancers (Martinez-Outschoorn et al., 2011; Nencioni et al., 2013; Chang et al., 2014). In the present study, we recognized autophagy induction by the cisplatin treatment in cervical cancer HeLa cells. Treatment with 0.2 μg/mL cisplatin significantly promoted accumulation of autophagy-specific vesicles, i.e. acidic vesicular organelles (AVOs) (Paglin et al., 2001) in HeLa cell cytosol, which were confirmed by GFP-LC3 labeled autophagosomes and morphologic observation of autophagic vesicles. Additionally, protein expression of ATG7, LC3-I, and LC3-II were also examined, all of which play important roles in autophagosome formation (Daido et al., 2004). We further confirmed that the autophagy inhibitor, 3MA, could inhibit cisplatin-induced autophagy in the HeLa cells. Our results showed reduced AVO formation, reduced conversion of LC3-I to LC3-II, and of reduced ATG7 expression in the HeLa cells subject to both 3MA and cisplatin.

Moreover, we confirmed that autophagy inhibition by 3MA sensitized cervical cancer cells to cisplatin. According to MTT assay results, viability of HeLa cells post-treatment with both 3MA and cisplatin was significantly reduced as compared to cells treated with only 3MA or cisplatin. Such significant difference between the combined treatment and the singular treatment with either

3MA or cisplatin was also confirmed in apoptosis induction of HeLa cells. Thus, we confirmed that blocked autophagy by 3MA sensitizes cervical cancer cells to cisplatin treatment.

In summary, the present study recognized autophagy induction by cisplatin in cervical cancer HeLa cells. Such autophagy induction might be an adaptive response of cervical cancer cells to chemotherapy. In addition, autophagy blockage sensitizes cervical cancer cells to the chemotherapy.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENT

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