RESEARCH ARTICLE

Effect of Autophagy-Related Beclin1 on Sensitivity of Cisplatin-Resistant Ovarian Cancer Cells to Chemotherapeutic Agents

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Abstract

The purpose of the study was to determine the effects of autophagy related gene Beclin1 at different levels of expression on the sensitivity of cisplatin-resistant ovarian cancer cells (SKOV3/DDP) to different chemotherapeutics. In pSUPER-Beclin1 transfected cells, real-time fluorescence quantitative RT-PCR and Western blot analysis showed that expression was significantly inhibited. Flow cytometry revealed that the mean fluorescence intensity (MDC), reflecting autophagy, and cells in the G0/G1 phase were markedly reduced. When compared with the blank control group, inhibition of Beclin1 expression in SKOV3/DDP cells not only increased the rate of apoptosis following treatment with chemotherapeutics, but also increased the sensitivity. These findings suggest that Beclin1 expression plays an important role in chemotherapeutic agent-induced death of SKOV3/DDP cells. Inhibition of autophagy related gene Beclin1 expression in SKOV3/DDP cells may increase the rate of apoptosis and elevate the sensitivity to chemotherapeutics.

Keywords: Autophagy - apoptosis - beclin1 - cisplatin - ovarian cancer

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Introduction

Ovarian cancer is one of the most common malignancies in women. Patients with early stage ovarian cancer are usually asymptomatic, thus ovarian cancer has become the leading cause of death among malignancies of the reproductive system in women. Platinum-based chemotherapy with paclitaxel is an important adjunctive therapy for ovarian cancer, but some patients are insensitive to first-line chemotherapeutics. In addition, a fraction of ovarian cancer patients may develop recurrence due to secondary chemoresistance. Chemoresistance significantly limits the wide application of chemotherapeutics.

Programmed cell death functions to selectively remove useless or dangerous cells in multicellular animals. According to morphological features, programmed cell death can be divided into type I apoptosis and type II autophagy. Autophagy and apoptosis are both cellular degradation pathways essential for organismal homeostasis (Green et al., 2014). Therefore, it is not surprising that both autophagy and apoptosis have been implicated in protecting organisms against a variety of diseases, especially cancer (Liu and Ryan, 2012). Our previous studies (Sun et al., 2011) showed that the autophagy-related gene, Beclin 1, inhibits proliferation and invasion of cervical cancer cells in vitro and lead to the death of these cells, thus exerting an antitumor effect.

Studies have confirmed that cisplatin (DDP), a first-line chemotherapeutic for ovarian cancer, induces apoptosis and is a major mechanism underlying the anti-tumor effect of DDP; however, long-term chemotherapy with DDP may induce resistance and result in reduced sensitivity of cancer cells to apoptosis, which is a major cause of uncontrolled progression of cancers. Thus, to elevate the sensitivity of ovarian cancer cells to DDP-induced apoptosis has been a strategy to reverse chemotherapy-resistance. It is important to identify novel targets and develop new therapeutic strategies on the basis of non-apoptotic death, which has been a hot topic in studies on cancer therapy.

Materials and Methods

Materials and reagents

Human ovarian cancer SKOV3/DDP cells were purchased from the Cancer Hospital of Beijing. The cell lines were resistant to cisplatin and were prepared by repeated and intermittent treatment with cisplatin. RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA), G418, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), rabbit anti-human Beclin1 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA), horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and AnnexinV/PI apoptosis detection kit (KeyGEN Biotech, Nanjing, China) were used in the present study.

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Cisplatin, gemcitabine, ifosfamide, and docetaxel were supplied by Qilu Biotech (Jinan, China). Two plasmids encoding the human Beclin1 gene, pcDNA3.1-Bec (overexpression construct) and pSUPER-Bec (partial-silencing construct), were assembled and kindly provided by the Laboratory of Gynecologic Oncology, West China Second Hospital of Sichuan University. Other reagents were of analytic grade.

Culture of SKOV3/DDP cells and transfection

The SKOV3/DDP cells were grown in RPMI-1640 supplemented with heat inactivated 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO_2. Cells in the logarithmic phase were digested with trypsin and then seeded in a 6-well plate (5×10^5/well). One day later, pcDNA3.1-Bec and pSUPER-Bec were independently used to transfect the cells using lipofectamine 2000 according to the manufacturer's instructions. One day after transfection, G418 was used to screen for positive colonies. The pcDNA3.1-Bec vector presents green fluorescence, and cells carrying green fluorescence were regarded as positive, indicating successful transfection. One week later, positive colonies were obtained and cells were resuspended in medium followed by culture to achieve cells with stable expression of Beclin1. Cells successfully transfected with pcDNA3.1-Bec or pSUPER-Bec were defined as the pcDNA3.1-Bec group and pSUPER-Bec group, respectively. In addition, cells transfected with pcDNA3.1 (empty vector) or pSUPER (scramble RNA) were defined as the pcDNA3.1 group and pSUPER group, respectively. SKOV3/DDP cells without transfection served as a blank control. The recombinant vectors were confirmed by digestion analysis using restriction endonucleases, and all the constructed plasmids were confirmed by DNA sequencing.

Western blot analysis of Beclin1

After stable transfection, the attached SKOV3/DDP cells were washed twice with PBS, harvested in PBS, and pelleted by centrifugation. The pellet was weighed and suspended in 2× Laemmli buffer. Fifty micrograms of protein was loaded on a 15% sodium dodecyl sulfate polyacrylamide gel, and transferred to nitrocellulose. After blocking, membranes were incubated for 1 h with anti-Beclin1 antibody, followed by a horseradish peroxidase-conjugated anti-rabbit IgG antibody. Specific bands were detected by an enhanced chemiluminescence system (Pierce, Rockford, IL, USA). Anti-actin was used to ensure equal loading. Band intensity was semi-quantified using Photoshop Elements software after conversion to digitalizing image using an image scanner (GT9700F; Epson, Tokyo, Japan).

RT-PCR detection of Beclin1 mRNA

Total RNA was extracted using Trizol, and 2 μg of RNA was used for reverse transcription and polymerase chain reaction(PCR). These experiments were performed in triplicate. The primers used were as follows: Beclin1, 5'-AGGAACTCACAGCTCCATTAC-3' (forward) and 5'-TTGAGTCCTCTCCTCGGTAA-3' (reverse), with an anticipated size of 316 bp. β-actin served as an internal reference, and the primers used were as follows: 5'-GAAGATCAAGATCATTGCTCCT-3' (forward) and 5'-TACTCCTGCTTGCTGATCCA-3' (reverse), with an anticipated size of 311 bp. The conditions for PCR were as follows: pre-denaturation at 94ºC for 2 min; 45 cycles of denaturation at 94ºC for 20 s, annealing at 55ºC for 30 s, and extension at 60ºC for 40s; and a final extension at 72ºC for 5 min. The increase in fluorescence intensity was measured and used to delineate the amplification curve, according to which the Ct value (Ct value reflects the fluorescence intensity) and △Ct (Ctsample-Ctβ-actin) were determined, followed by analysis using the $2^{-\triangle\triangle Ct}$ method. The expression of the target genes was normalized to that in the cells transfected with an empty control plasmid. The data represented the relative expression of the target genes.

Detection of apoptosis following Hoechst 33258 staining

Cells in the logarithmic phase were used to prepare a cell suspension (3×10^5 cells/ml), and this was then seeded into 6-well plates (2 ml/well), followed by incubation at 37°C in 5% CO_2 for 48 h. These cells were then fixed in 4% paraformaldehyde for 15 min and washed with PBS. Subsequently, 0.5 ml of Hoechst 33258 was added to each well, followed by incubation at room temperature for 4 min. After washing with PBS, the cells were observed under a fluorescence microscope.

Analysis of the cell cycle

The untransfected or stably transfected cells were harvested by trypsinization, fixed with cold 70% ethanol, and stored at 4°C until analyzed. The cells were resuspended in 100 μL of RNaseA (180 μg/mL) and incubated at room temperature for 30 min. Propidium iodide (final concentration, 50 μg/mL; Merck, Darmstadt, Germany) was added, and cells were incubated at room temperature in the dark for 15 min, and DNA content was detected by FACScan™ flow cytometry system (Becton Dickinson, San Jose, CA, USA). The relative proportions of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined from the flow cytometry data.

Quantification of MDC labeling of cells

To measure the autophagic ratio, transfected cell pellets were suspended with 0.05 mmol/L MDC (the autophagosome-specific dye monodansylcadervarine; Sigma) at 37°C for 60 min. The cellular fluorescent changes were observed using OLYMPUS IX70 reverse fluorescent microscopy (Olympus, Tokyo, Japan) equipped with Motic Image Advanced 3.0 software as collective system. The samples were also analyzed on a FACScan™ flow cytometry system. Data were analyzed using winMDIv2.8 software to determine the percentage of cells undergoing autophagy, i.e., those cells that have recruited MDC-positive particles. All experiments were performed at least three times for each experimental condition.

MTT-assay for the drug sensitivity

The sensitivity of the stably transfected cells to

cisplatin, gemcitabine, ifosfamide, and docetaxel was detected with a MTT assay. Cells (5,000) were cultured in each well in a 96-well plate for 24 h. The culture medium was replaced with the medium containing serial dilutions of various chemotherapeutic drugs. After 48 h of drug incubation, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, 5 mg/ml; Sigma) was added to each well and incubated for an additional 4 h. The plates were then centrifuged (500 x g, 10 min) and the supernatant was removed. Dimethyl sulfoxide (DMSO, 100 μl/well; Sigma) was added to dissolve the blue formazan crystals converted from MTT by live cells. Cell viability was assessed by absorbance at 570 nm measured on a μQuant Universal Microplate spectrophotometer (Spectra Max model 190; Molecular Devices, Sunnyvale, CA, USA). The absorbance (A) values of formazan were calculated as a percentage of the control untreated wells, and transferred to a dose-response curve. The survival rate of tumor cells to each drug with different concentrations was calculated as follows: survival rate = 100% × (Adrug treated-Ablank)/(Acontrol-Ablank). The IC50 value resulting from 50% inhibition of cell growth was calculated. Each concentration of drugs was measured in triplicate wells on the same plate in three independent experiments.

Flow cytometry analysis of cell apoptosis

After 48 h of drug incubation, the cells were simultaneously stained with fluorescein isothiocyanate (FITC)-labeled annexin-V and PI stain, according to the IC50 value of different drugs, to discriminate viable cells (low FITC and low PI signal) from early apoptotic cells (high FITC and low PI signal) and cells that had lost membrane integrity as a result of very late apoptosis (high FITC and high PI signal). A total of 1.0×10^6 stably transfected SKOV3/DDP cells were washed twice with ice cold PBS and incubated for 30 min in a binding buffer (1 μg/ml PI and 1 μg/ml FITC-labeled annexin-V), respectively. FACS analysis for annexin-V and PI staining was performed by the above-mentioned flow cytometer. All experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software. Studies were performed in triplicate with the results expressed as the mean ± SD as appropriate. Results were considered statistically significant at a $p<0.05$ obtained with a two-tailed Student's t-test.

Results

Beclin1 expression in transfected cells

After recombinant plasmid-transfected SKOV3/DDP cells and monoclonal cells with stable expression were screened, the cell transfection efficiency of a 200-fold fluorescence microscope l0 view cells was counted as the percentage of the total cells. Greater than 80% of the cells exhibited positive fluorescent signals (green fluorescent protein [GFP]) under an inverted fluorescence microscope. To determine the influence of Beclin1 transfection of SKOV3/DDP cells, RT-PCR and Western blotting were performed to analyze Beclin1 expression changes. After RT-PCR and delineation of the kinetic curves following PCR amplification, the relative expression of Beclin1 was calculated. As shown in Table 1, Beclin1 expression in the pcDNA3.1-Bec group was significantly higher than in the blank control group. However, Beclin1 expression in the pSUPER-Bec group was significantly decreased when compared with the blank control group. Western blot analysis also showed that the pcDNA3.1-Bec group had increased Beclin1 expression and that the pSUPER-Bec group effectively inhibited the protein expression of Beclin1 (Figure 1).

Effect of Beclin1 on the morphology of apoptotic SKOV3/DDP cells

Following Hoechst 33258 staining, apoptotic cells were characterized by dark blue fluorescence, condensation and margination of chromatin, and a patchy or shrunken/ruptured nucleus. In the blank control group and pSUPER-Bec group, the SKOV3/DDP cells mainly presented with even blue fluorescence and apoptotic cells were only occasionally observed. However, the number of apoptotic cells was significantly increased in the pcDNA3.1-Bec group (Figure 2).

Effects of Beclin1 on the SKOV3/DDP cell cycle

In the current study, the effect of Beclin1 on the SKOV3/DDP cell cycle was determined and each assay was performed in triplicate. As shown in Table 2, the percentage of pcDNA3.1-Bec cells in the G0/G1 phase significantly increased to 62.5% compared with 48.7%

Table 1. Real-time Flourescent Quantitative RT-PCR for Detecting the Expression of Beclin1 mRNA ($\bar{x}\pm s$)

Cell	△Ct	$2^{-\triangle\triangle Ct}$
SKOV3/DDP	5.6±0.09	1.09±0.13
pcDNA3.1-Bec	0.8±0.02	33.19±2.66*
pcDNA3.1	5.8±0.08	0.98±0.12
pSUPER-Bec	7.5±0.11	0.36±0.02
pSUPER	6.1±0.07	0.87±0.08

Asterisks denote values that were significantly different from the SKOV3/DDP cells ($p<0.01$)

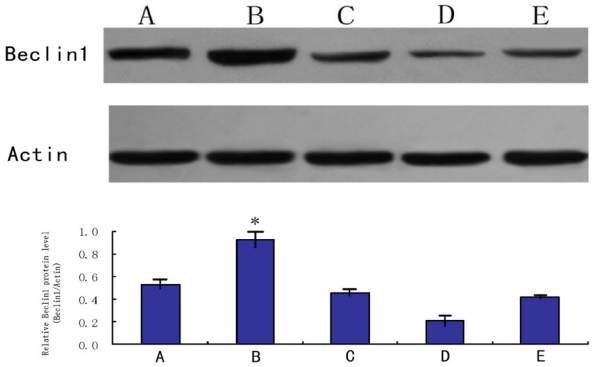

Figure 1. Beclin1 Protein Expression were Analyzed Using Western Blotting. Data are the mean±SD; *$p<0.05$ compared with the blank control SKOV3/DDP cells or pSUPER-Bec cells. A: SKOV3/DDP; B: pcDNA3.1-Bec; C:pcDNA3.1; D: pSUPER-Bec; E: pSUPER

of the non-transfected cells, while the percentage of pcDNA3.1-Bec cells in the S phase significantly decreased to 25.2% compared to 38.3% of the non-transfected cells (P<0.05), which indicated that overexpression mediated up-regulation of Beclin1 expression-induced cell cycle arrest in the G0/G1 phase.

Detection of autophagy by flow cytometry

After absorbing MDC, the fluorescent dye, cells selectively gather in autophagosome. Quantity of the formed autophagosome can be known by testing fluorescence intensity with flow cytometry. The flow cytometric analysis indicated that the percentage of MDC-positive cells was larger in the pcDNA3.1-Bec group than that in the non-transfected group, and the proportion of autophagic cells increased from 7.1% in the non-transfected group to 33.6% in the pcDNA3.1-Bec group (Figure 3). These results suggest that autophagy was enhanced in Beclin1 over-expressed SKOV3/DDP cells.

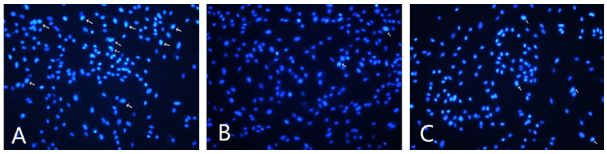

Figure 2. Effect of Beclin1 on SKOV3/DDP Cells by Hoechst 33258 Staining: A. pcDNA3.1-Bec; B. pSUPER-Bec; C. Untransfected SKOV3/DDP cells. pcDNA3.1-Bec transfectant resulted in plentiful apoptosis in contrast to pSUPER-Bec transfectant and control agents. Representative fields are shown and apoptotic cells are indicated by arrows

Table 2. Effect of Beclin1 on the Cell Cycle of SKOV3/DDP Cells

Cell	Cell cycle (mean±SD%)		
	G0/G1	S	G2/M
SKOV3/DDP	48.7±1.5	38.3±1.2	10.1±1.1
pcDNA3.1-Bec	62.5±2.3*	25.2±0.3*	10.7±0.5
pcDNA3.1	49.9±1.6	35.2±0.8	10.9±1.1
pSUPER-Bec	43.1±1.2	41.9±1.6	13.7±1.5
pSUPER	47.4±0.6	38.7±0.8	12.3±0.5

Asterisks denote values that were significantly different from the SKOV3/DDP cells (p<0.05)

Knockdown of Beclin1 does increase chemosensitivity in SKOV3/DDP cells

To test if the autophagy-related gene, Beclin1, plays a role in the regulation of chemosensitivity to anti-cancer drugs, we examined its effect on sensitivity to several common anti-cancer agents in SKOV3/DDP cells. The sensitivities of SKOV3/DDP cells to various concentrations of cisplatin, gemcitabine, ifosfamide, and docetaxel were determined by an MTT assay. Survival rates were plotted on curves (Figure 4). The IC50 values of different drugs are also given in Table 3. As shown in Table 3, the IC50 values of cisplatin, gemcitabine, ifosfamide, and docetaxel were significantly reduced in Beclin1 siRNA clones, compared to the Scramble RNA -transfected cells (p<0.01). There was a consistent increase in drug sensitivity in Beclin1 siRNA clones for each agent tested. The Beclin1 siRNA increased sensitivity of SKOV3/DDP to these drugs.

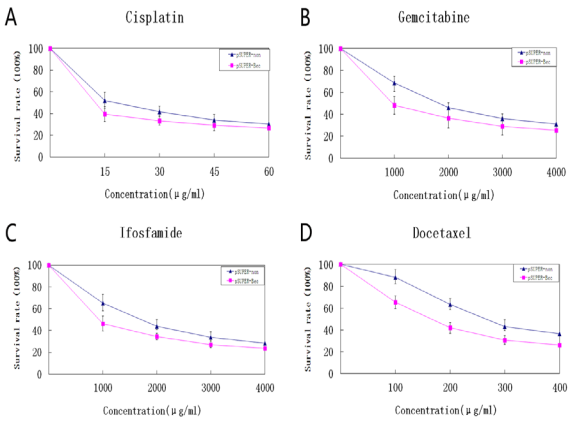

Figure 4. Enhanced Sensitivity to Chemotherapeutic Drugs in pSUPER-Bec SKOV3/DDP Cells. Cells were incubated with the indicated concentrations of cisplatin (A), gemcitabine (B), ifosfamide (C), and docetaxel (D) for 48 h, and cell viability was measured by the MTT assay. pSUPER-Bec SKOV3/DDP cells have more sensitivity to these drugs than vector-only controls. It means the statistics significance with the code. Data values are the mean±SD

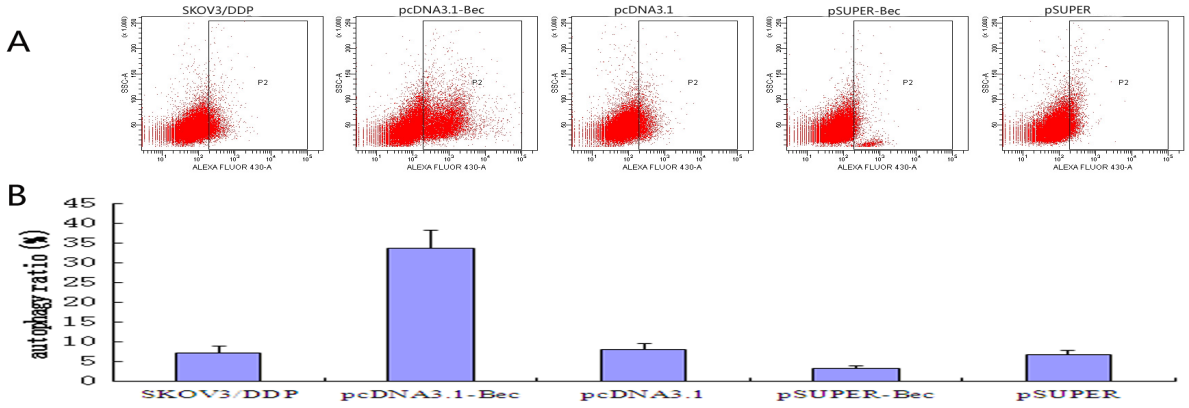

Figure 3. A. Autophagy was Determined by Flow Cytometry for MDC Labeling. Percentage of autophagic cells is in gate P2. B. The proportion of autophagic cells was analyzed by flow cytometry. The autophagic rate of pcDNA3.1-Bec cells significantly increased to 33.6%, compared with 7.1% of non-transfected cells (P<0.05). Data (% of the total population) are from at least three replicate experiments

Table 3. Effect of Beclin1 siRNA on Sensitivity of SKOV3/DDP Cells to Chemotherapeutic Drugs

Drugs	IC50 value (μg/ml)	
	pSUPER	pSUPER-Bec
Cisplatin	15.32±2.53	10.13±1.39
Gemcitabine	1363.85±85.27	916.62±36.35
Ifosfamide	1252.73±117.63	871.76±99.26
Docetaxel	256.73±19.32	168.61±16.11

Asterisks denote values that were significantly different from the control cells (p<0.01). Cell survival was determined by MTT assay. The IC50 value resulting from 50% inhibition of cell growth was calculated. Each value represents the means ± SD of three independent experiments

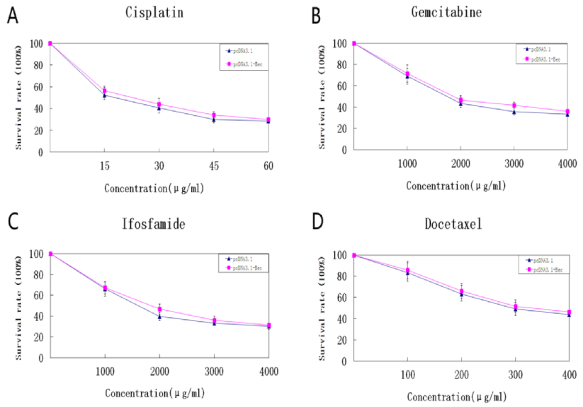

Figure 5. Do not Enhanced Obviously Resistance to Chemotherapeutic Drugs Correlated with Beclin1 Overexpression in SKOV3/DDP Cells. SKOV3/DDP cells were stably transfected with pcDNA3.1-Bec or positive control pcDNA3.1, then the cells were exposed for 48 h at the indicated concentrations to cisplatin (A), gemcitabine (B), ifosfamide (C), and docetaxel (D). Cell viability was determined by the MTT assay. Although overexpression of Beclin1 leads to more resistance to these drugs than vector-only controls, the difference was not significant (p>0.05). Data are the mean±SD

Overexpression of Beclin1 does not lead to increase drug resistance in SKOV3/DDP cells

Having demonstrated that Beclin1 siRNA further sensitizes SKOV3/DDP cells to chemotherapeutic agents, we attempted to determine whether increased Beclin1 expression would render SKOV3/DDP cells more resistant to these drugs. SKOV3/DDP cells were stably transfected with the synthetic pcDNA3.1-Bec or pcDNA3.1 followed with the drug treatments for a further 48 h before cell viability was measured using the MTT assay. Survival rates were plotted on curves (Figure 5). The IC50 values of different drugs are also given in Table 4. As shown in Table 4, compared with vector control, the SKOV3/DDP cells transfected with the synthesized pcDNA3.1-Bec demonstrated increased IC50 values of cisplatin, gemcitabine, ifosfamide, and docetaxel, but the differences were not significant (p>0.05).

Effect of Knockdown Beclin1 on anti-cancer drug-induced apoptosis

To confirm the augmentation of cisplatin-, gemcitabine-, ifosfamide-, and docetaxel-induced apoptosis by Knockdown Beclin1 in SKOV3/DDP cells, annexin-V staining assays were performed. Chemotherapeutic drugs alone- or chemotherapeutic drugs plus pSUPER-Bec

Table 4. Effect of Beclin1 Overexpression on Sensitivity of SKOV3/DDP Cells to Chemotherapeutic Drugs

Drugs	IC50 value (μg/ml)	
	pcDNA3.1	pcDNA3.1-Bec
Cisplatin	18.11±3.86	20.63±3.77
Gemcitabine	1396.28±110.73	1490.17±101.96
Ifosfamide	1247.45±124.55	1257.36±111.63
Docetaxel	286.96±36.16	301.91±20.63

Cell survival was determined by MTT assay. The IC50 value resulting from 50% inhibition of cell growth was calculated. Each value represents the means±SD of three independent experiments

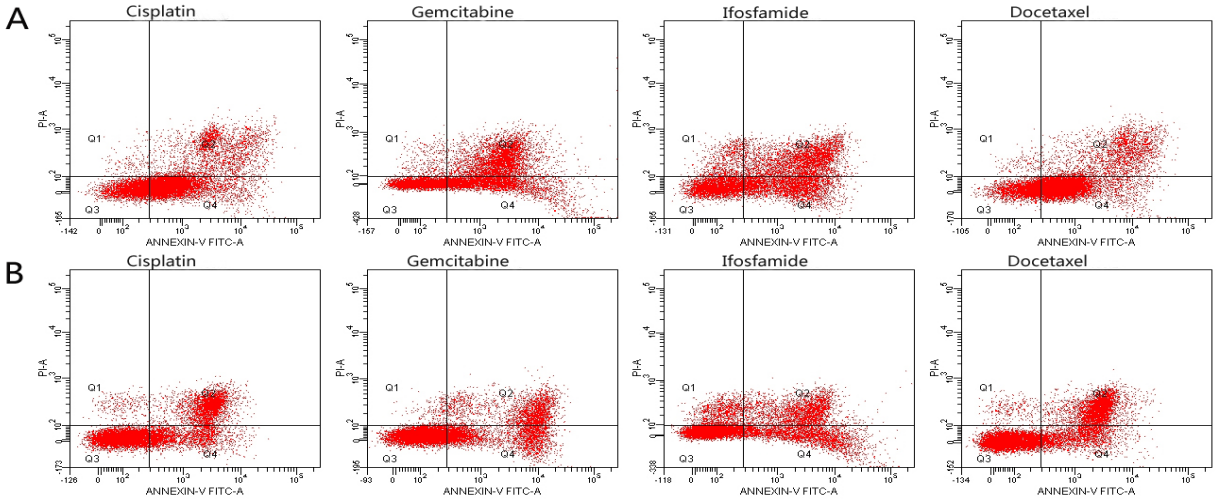

Figure 6. Apoptosis was Determined by flow Cytometry for Annexin-V-FITC and Propidium Iodide (PI) Dual Labeling. Cytograms of annexin-V-FITC binding (abscissa) versus PI uptake (ordinate) show three distinct populations: *i*) viable cells in gate Q3; *ii*) early apoptotic cells in gate Q4; *iii*) cells that have lost membrane integrity as a result of very late apoptosis in gate Q2. Percentage of apoptotic cells is gate Q4 plus gate Q2. After 48 h of drug incubation, according to the IC50 values of cisplatin, gemcitabine, ifosfamide, and docetaxel, the apoptotic rate of pSUPER-Bec cells (A) significantly increased to 72.3%, 66.5%, 62.1%, and 75.6%, compared with 48.6%, 46.2%, 44.5%, and 48.3% of non-transfected cells (B; P<0.05). Data (% of the total population) are from at least three replicate experiments

induced apoptosis analysis was performed with annexin-V/PI staining using the Apoptosis Assay Kit, and the results showed that the combination of chemotherapeutic drugs and pSUPER-Bec induced significant increases in apoptosis over that of each agent alone in SKOV3/DDP cells (Figure 6). Taken together with our MTT assay, these results suggest that a combination of Beclin1 siRNA biotherapy and anti-cancer drug chemotherapy is potentially useful as a novel therapeutic approach for treating ovarian carcinoma in the clinic.

Discussion

Autophagy is important for the maintenance of balance between synthesis, degradation, and recycling of cellular components. Autophagy involves the sequestration of portions of the cytoplasm within double-membrane vesicles (autophagosomes), which fuse with lysosomes to form autolysosomes in which autophagic cargo is degraded (Choi et al., 2013). This catabolic process, which is mediated by a number of autophagy and autophagy-related proteins, is primarily used for cellular adaptation to dwindling nutrient resources and is generally considered a protective response. However, in some cases it may also participate in cell death (Denton et al., 2012), and it has sometimes been viewed as a separate modality of programmed cell death. It is generally thought that autophagy has two primary and opposing functions in tumor cells in response to stress induced by chemotherapy. One is the cytoprotective function that can in theory be inhibited for therapeutic advantage by sensitizing the cells to these treatment modalities. The other is the cytotoxic function that is generally not observed with conventional treatment modalities, but that may function to promote tumor cell killing either alone or in association with apoptosis (Gewirtz et al., 2014). In recent years, studies have also revealed that autophagy has a complex relationship with apoptosis, both of which are types of programmed cell death (Pandey et al., 2012). Autophagy may induce apoptosis and is an essential factor for cell death. Cell apoptosis and autophagy exist independently, and there might be a molecular switch regulating autophagy and apoptosis, and determining cell fate (Zhang et al., 2014).

The Beclin1 gene encodes the Beclin1 protein, which is a well-established regulator of the autophagic pathway. The Beclin1 gene is mapped to 17q21, encodes a sequence with 450 amino acids and 60 kDa in molecular weight. It is a mammalian orthologue of the ATG6 gene in yeast and was one of the first identified mammalian autophagy-associated genes. Beclin1 interacts with a number of binding partners in the cell which can lead to either activation (eg, via PI3KC3/Vps34, Ambra 1, UV radiation resistance-associated gene) or inhibition (eg, via Bcl-2, Rubicon) of the autophagic pathway (Sahni et al., 2014). Apart from its role as a regulator of autophagy, it is also shown to effect important biological processes in the cell such as apoptosis and embryogenesis (Han et al., 2014; Sun et al., 2014; Wang et al., 2014). In the present study, Beclin1-siRNA plasmids (pSUPER-Bec) and Beclin1-overexpressing plasmids (pcDNA3.1-Bec) were independently transfected into DDP-resistant ovarian cells (SKOV3/DDP cells). Western blot analysis and RT-PCR confirmed that Beclin1 expression in the pSUPER-Bec group was significantly lower than non-transfected cells, and pcDNA3.1-Bec was markedly higher than non-transfected cells. In the pcDNA3.1-Bec group, flow cytometry revealed that autophagy was increased in SKOV3/DDP cells and Hoechst33258 staining revealed that the apoptosis rate increased. These findings suggest that Beclin1 overexpression in SKOV3/DDP cells may not only elevate autophagy, but promote apoptosis. Cell proliferation and apoptosis are closely related to cell cycle. In the present study, flow cytometry further revealed that Beclin1 overexpression could arrest more cancer cells in the G0/G1 phase when compared with the blank control group. We speculate that Beclin1 may induce cell autophagy and further arrest cells in the G0/G1 phase by affecting the repair of DNA injury, accumulating cell injury, and finally causing cell apoptosis.

It is well-known that apoptosis is crucial for chemotherapy-induced death of cancer cells (Meyn et al., 2009), and anti-tumor drugs may induce apoptosis of cancer cells. Although autophagy and apoptosis are under the control of multiple common upstream signals, these processes also cross-regulate each other, mostly in an inhibitory manner. Thus, autophagy reduces the propensity of cells to undergo apoptosis, and activation of the apoptotic programme is coupled to the suppression of autophagy (Mariño et al., 2014). In recent years, autophagy regulators have been used in combination with chemotherapeutics for targeted therapy of cancers. There is evidence (Cui et al., 2007) showing that rubescensin, a Traditional Chinese Medicine with anti-tumor activity, may induce and increase the autophay of cervical cancer cells (HeLa cells), and 3-methyladenine(3MA), an inhibitor of autophagy, may increase the anti-tumor effect of rubescensin. In studies on malignancies, such as leukemia, the results have revealed that 3MA is able to effectively increase the therapeutic efficacy of some chemotherapeutics (Palmeira et al., 2014). In addition, studies have shown that the combined use of the autophagy inhibitor, CQ, may increase the sensitivity of liver cancer cells to 5-fluorouracil (Guo et al., 2012), and sorafenib in combination with CQ may increase the endoplasmic reticulum stress-induced death of liver cancer cells in vivo and in vitro (Shi et al., 2011).

On the basis of the above findings, this study was conducted to alter Beclin1 expression in SKOV3/DDP cells, and cell autophagy and the sensitivity to chemotherapeutics were further investigated. In the pilot study, we determined the IC50 of cisplatin in cells transfected with blank vector and Beclin1 siRNA after treatment with cisplatin for 12, 24, 36, and 48 h. The results showed that the IC50 at different time points after cisplatin treatment in cells transfected with Beclin1 was significantly lower than cells without transfection, and the lowest IC50 was observed at 48 h. Thus, IC50 at 48 h was used in the following experiments. Our results showed that following chemotherapeutic treatment the rate of apoptosis in SKOV3/DDP cells transfected with Beclin1 siRNA increased significantly when compared

with the blank control group. In the presence of Beclin1 overexpression, however, the chemotherapeutic agent-induced apoptosis was reduced, although a significant difference was not observed. These findings suggest that Beclin1 plays pivotal roles in the chemotherapeutic agent-induced death of SKOV3/DDP cells, and active autophagy of SKOV3/DDP might be ascribed to the resistance to chemotherapeutics. Cancer cells may clear metabolic byproducts via autophagy and increase their tolerance to environmental change, resulting in reduced sensitivity to chemotherapeutics. Under these conditions, to interfere with Beclin1 expression may inhibit cell autophagy and increase the chemotherapeutic agent-induced apoptosis of SKOV3/DDP cells. Our in vitro studies further revealed that the proliferation of SKOV3/DDP cells transfected with Beclin1 siRNA was markedly slowed when compared with cells transfected Beclin1-overexpressing plasmids and cells in the blank control group. This suggests that interfering with Beclin expression in combination with chemotherapeutic has a better anti-tumor effect than monotherapy. This also indicates that cell autophagy and apoptosis, two types of programmed cell death, are involved in chemotherapeutic agent-induced death of cancer cells. Although there are differences in the molecular mechanisms between autophagy and apoptosis, there might be a switch between autophagy and apoptosis of SKOV3/DDP. Under certain conditions, to interfere with autophagy and apoptosis may increase the sensitivity of cancer cells to chemotherapeutics; however, additional studies are warranted to further investigate the specific mechanism, and the dose and timing of chemotherapeutics in gene therapy in-depth.

Taken together, autophagy plays an important role in the occurrence, development, and therapy of cancer, and the chemotherapy-resistance of cancer cells is closely related to the autophagy of cancer cells. Autophagy may be actually populated by at least two additional players. One have been termed the nonprotective form of autophagy, where the cell is apparently carrying out autophagy-mediated degradative functions, but where autophagy inhibition does not lead to perceptible alterations in drug sensitivity. The other is what we now term the cytostatic form of autophagy in that its activation results in prolonged growth inhibition as well as reduced clonogenic survival but in the absence of actual loss of cell viability through apoptosis; however, as is the case with cytototoxic autophagy, inhibition of cytostatic autophagy protects the tumor cell from the agent that promotes the autophagic response. Our results showed that inhibition of Beclin1 expression may suppress cell autophagy and promote apoptosis, which increases the sensitivity of cancer cells to chemotherapeutics. On the basis of these findings, we suggest that additional studies are required to elucidate the molecules and signaling pathways involved in autophagy and apoptosis, which may further explain the relationship between autophagy and chemosensitivity of cancer cells and provide a new strategy for targeted therapy of ovarian cancer via combining autophagy-modulating therapy and chemotherapy.

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