Autophagy inhibition promotes paclitaxel-induced apoptosis in cancer cells

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Abstract
Paclitaxel has been demonstrated to be an effective mitotic inhibitor and apoptosis inducer to treat aggressive malignancies. In this paper, we have provided a line of evidence that promotion of apoptotic cell death by paclitaxel was accompanied with induction of autophagy in A549 cells. Paclitaxel treatment could lead to the formation of acidic vesicular organelles (AVOs), the induction of Atg5, Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3) expressions, and the increase of punctate fluorescent signals in A549 cells pre-transfected with green fluorescent protein (GFP)-tagged LC3. Interestingly, paclitaxel-mediated apoptotic cell death was further potentiated by pretreatment with autophagy inhibitor 3-methyladenine (3-MA) or small interfering RNA against the autophagic gene beclin 1. These findings suggest that paclitaxel-elicited autophagic response plays a protective role that impedes the eventual cell death, and inhibition of autophagy could be an adjunctive strategy for enhancing chemotherapeutic effect of paclitaxel as an antitumor agent.

1. Introduction
Chemotherapy is an important option in curing or controlling various cancers including lung cancer. Paclitaxel, which stabilizes microtubule and causes apoptosis, offers both symptomatic and survival benefits for lung adenocarcinoma. The paclitaxel-based combination therapies are standard treatments for nearly all patients diagnosed with non-small cell lung carcinoma (NSCLC) [1]. However, clinical treatment with paclitaxel often encounters a number of undesirable side effects as occurred using other anticancer agents. The dose increment of systemic administration of paclitaxel would generate unacceptable levels of toxicity to normal cells, especially of bone marrow, gastrointestinal tract, and the hair follicles [2]. Therefore, many attempts have been made to enhance its therapeutic effectiveness, simultaneously reducing its toxicity. In an effort to search for strategies that could enhance cancer cell killing mediated by paclitaxel, we have investigated possible pro-survival pathways that are activated in response to paclitaxel. Herein, we report the induction of autophagy by paclitaxel.

Autophagy is an evolutionary conserved process in which cell engulfs cytoplasmic constituents within a double-membrane vacuole (named autophagosome) and delivers them to the lysosome for degradation [3].

Abbreviations: AVOs, acidic vesicular organelles; LC3, microtubule-associated protein 1 light chain 3; 3-MA, 3-methyladenine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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Autophagy contributes to maintaining cellular homeostasis as a result of quality control of both proteins and organelles. In addition to its basic role in the turnover of proteins and organelles, autophagy has multiple physiological and pathophysiological functions [4,5]. When cells encounter environmental stressors such as nutrient starvation and pathogen infection, autophagy is induced to provide nutrients and energy required for cell survival. So autophagy is recognized as a cytoprotective process against environmental stress [6,7]. Meanwhile, autophagy is also an alternative route of programmed cell death, called type-2 programmed cell death or autophagic cell death [8]. In tumor cells, the role of autophagy may depend on the type of tumor, the stage of tumorigenesis, and the nature and extent of the insult. Appropriate modification of autophagy, that is, inhibition of cytoprotective autophagy or promotion of cyto-killing autophagy could augment cytotoxicity caused by anticancer therapy in tumor cells [9–11]. Thus, in addition to apoptotic response, it would be very useful to determine if an antitumor agent can induce autophagy and what type of autophagy it is. In this study, we found that paclitaxel-induced autophagy in cancer cells, and inhibition of autophagy could lead to enhancement of paclitaxel-mediated cytotoxicity through increasing apoptosis.

2. Materials and methods

2.1. Cell lines and cell culture

A549, PC-3, and HT-29 cell lines were obtained from The Cell Bank of Chinese Academy of Sciences (Shanghai). Human glioma cancer cell line U87 which stably expresses GFP-LC3 protein was kindly gifted by Yan bing (Shan Dong university). A549 cells were cultured with F12 medium supplemented with 10% foetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), PC-3, HT-29 and GFP-LC3 transfected U87 cells were maintained in DMEM medium supplemented with 10% foetal bovine serum and antibiotics. Cultures were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

2.2. Cell viability measurement

Cell viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay as described previously [12]. Cells were seeded in 96-well plates at a density of 1 × 10⁴ cells and incubated for 24 h. After treatment with paclitaxel (10, 20, 40 nM, respectively) in the presence or absence of 3-MA (5 mmol/L), 10 μL of MTT (5 mg/mL) was added to each well and incubated in a humidified 5% CO₂ atmosphere at 37 °C for 4 h. Crystals were dissolved in 100 μL of DMSO. The absorbance of the solution was read spectrophotometrically at 570 nm using a microtitre plate reader (Bio-Rad). Cell viability was calculated according to the following formula: cell viability (%) = A570 (tested group-blank group)/A570 (vehicle-treated control group-blank group) × 100. At least three replicates were performed for each treatment.

2.3. DAPI staining

Morphological changes of apoptosis were determined by DAPI staining as described previously [12]. A549 cells were treated with vehicle or desired concentrations of paclitaxel for 24 h. After washing with PBS, cells were fixed with methanol/aceton (1:1) for 5 min at room temperature. The fixed cells were then washed with PBS and permeabilized with 0.1% TritonX-100 for 10 min prior to staining with DAPI (1:2000 dilution, in 1x PBS) for 10 min. The cells were washed with PBS and mounted. Images of DAPI fluorescence were collected using a Nikon phase-fluorescence microscope. Moderately fluorescent and round nuclei were considered normal. Bright and condensed/fragmented nuclei were regarded as apoptotic.

2.4. Immunoblotting assay

After treatment with paclitaxel at desired concentrations, cells were lysed with a solution containing Tris–HCl (50 mmol/L, pH 6.8), SDS (2% w/v), glycerol (10%), and dithiothreitol (10 mmol/L), supplemented with protease inhibitor mix (Thermo Fisher). Cell lysates were centrifuged at 12,000g for 30 min. Equal amounts of the protein
(50 μg) were resolved by SDS–PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in TBS for 1 h at room temperature and then incubated with primary antibodies against Bcl-2, Bax (Cell Signaling Technology), LC3, Atg5, Beclin 1 (Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) at 4°C overnight. Membranes were washed and treated with appropriate secondary antibodies for 1 h at room temperature. The immunocomplexes were detected with the enhanced chemiluminescence plus kit.

2.5. Detection of acidic vesicular organelles (AVOs)

Autophagy triggered by paclitaxel was examined by staining A549 cells with acridine orange (AO). Cells were plated on coverslips in 24-well plates and treated with paclitaxel (10, 20, 40 nM) for 9 h. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and then stained with AO (1 μg/mL in PBS) at 37°C in the dark. Images of AO staining were visualized immediately by Nikon phase-fluorescence microscope. To quantify the number of acidic vesicles in cells treated with paclitaxel, A549 cells were seeded into 6-well plates. After staining with AO in PBS at 37°C for 15 min, the cells were washed with PBS and analyzed by flow cytometry assay.

2.6. Involvement of LC3

A549, PC3 and HT-29 cells were plated in 24-well plates and transfected with a green fluorescent protein (GFP)-tagged LC3 expressing vector (1 μg/well) using the Lipofectamine 2000 (Invitrogen). After 24 h transfection, cells were exposed to paclitaxel for an additional 9 h. The punctate patterns of LC3 in transfected cells were examined by fluorescence microscopy. To detect autophagy in U87 cells which stably express GFP-LC3 protein, we plated U87-GFP-LC3 cells on coverslips in 24-well plates. After exposure to paclitaxel for 9 h, the fluorescent signals in cells were observed with fluorescence microscopy. Vehicle-treated cells served as a control.

2.7. RNA interference of beclin 1

A549 cells were seeded in 6-well plates and incubated overnight. A control random small interfering RNA (siRNA) or beclin 1-targeted siRNA (Invitrogen; 100 pmol/well) was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h transfection, cells were treated with paclitaxel for an additional 24 h. Then cells were collected and cell lysates were subjected to immunoblotting of Beclin 1 and LC3. Cells were also processed for cell viability and apoptosis analysis.
2.8. Apoptosis detection by flow cytometry

Cells were plated in six-well plates and incubated for 24 h. Then cells were exposed to desired concentrations of paclitaxel in the presence or absence of 3-MA. After 24 h of treatment, cells were harvested by trypsinization, washed twice in PBS. After staining with the combination of Annexin V/fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Annexin-V: FITC Apoptosis Detection Kit, BD Pharmingen), the cells were immediately analyzed by flow cytometer (FACS Calibur, Becton Dickinson).

3. Results

3.1. Paclitaxel induces apoptosis in A549 cells

In order to determine whether the observed cell death caused by paclitaxel was due to apoptosis, apoptosis parameters were analyzed by DAPI staining and Western-blot assay. As shown in Fig. 1A, nuclear morphological changes of apoptosis were observed in paclitaxel-treated cells. Paclitaxel caused significant increases in apoptotic cell number with condensed and fragmented DNA (as indicated by a strong blue fluorescence). In addition, alternations in the expression levels of Bax and Bcl-2 proteins also occurred after the paclitaxel treatment. As shown in Fig. 1B, treatment of A549 cells with 40 nM of paclitaxel for various time periods, the expression of Bax was up-regulated time-dependently, while Bcl-2 expression was down-regulated correspondingly. Furthermore, the native 116 kDa poly (ADP-ribose) polymerase (PARP) protein was cleaved into its characteristic 85 kDa fragment upon treatment with paclitaxel. These results supported the notion that paclitaxel induces apoptosis in A549 cells.

3.2. Paclitaxel triggers autophagy in A549 cells

Subsequently, we sought to determine whether treatment of cells with paclitaxel results in induction of autophagy in A549 cells. For that purpose, we initially determined a change in the expression level of the microtubule-associated protein 1 light chain 3 (LC3)-II, a marker for the presence of autophagosomes [13], in paclitaxel-treated A549 cancer cells by immunoblot analysis. As shown in Fig. 2A, the amount of LC3-II was found to be increased in cells exposed to paclitaxel as compared to a vehicle-treated control. An increase in the LC3-II was observed at 10 nM of paclitaxel and accumulated with the increasing concentrations of paclitaxel. Noteworthy, this concentration range was essential for paclitaxel to induce apoptosis in A549 cells. In addition, alterations in Atg5 and Beclin 1 which are also required for formation of autophagosomes were investigated in paclitaxel-treated cells [14,15]. Paclitaxel treatment led to increases in the expression levels of Atg5 and Beclin 1 in a dose-dependent manner in A549 cells as shown in Fig. 2A, suggesting that the treatment may cause an accumulation of autophagosome. Furthermore, the results in Fig. 2B showed that a significant accumulation in LC3-II expression was observed following 6 h exposure of paclitaxel, and then declined to basal level. However, paclitaxel induced Atg5 and Beclin 1 in A549 cells as early as 1 h and seemed to peak at 6 h.

In addition, paclitaxel-induced autophagic flux was also determined by transient transfection of cells with an expression plasmid containing GFP-LC3. Recruitment of LC3-II to the autophagosomes is characterized by punctate pattern of its localization. The vehicle-treated control cells (9 h treatment) exhibited diffuse and weak LC3-associated green fluorescence (Fig. 2C). However, the A549 cells treated with paclitaxel for 9 h exhibited characteristic punctate pattern of LC3. Consistent with the results in Fig. 2A, noticeable punctuate formation of GFP-LC3 appeared in cells treated with 10 nM paclitaxel and dose-dependently increased. Thus, these data indicate that paclitaxel was able to induce autophagosome formation in A549 cells.

3.3. Paclitaxel treatment induced formation of AVOs

We further confirmed the autophagic response to paclitaxel by analysis of acidic vesicular organelles (AVOs) formation. Paclitaxel treatment of A549 cells resulted in the increased formation of AVOs. Flow cytometry analysis showed that the number of acidic vesicles in A549 cells treated with paclitaxel increased in a dose-dependent manner, confirming the induction of autophagy (Fig. 3A and B). Similar results were also obtained on fluorescence microscopic examination. As shown in Fig. 3C, A549 cells
treated with paclitaxel for 9 h displayed a large number of fluorescent vesicles in the cytoplasm, whereas only few of fluorescent vesicles were observed in vehicle-treated cells.

3.4. Inhibition of autophagy by 3-MA potentiates paclitaxel-mediated apoptotic cell death

Since manipulation of autophagy has the potential to improve the efficacy of the anticancer therapeutics, we were eager to determine whether protective autophagy or autophagic cell death was elicited in the paclitaxel-mediated cancer cell death. The interconnection between paclitaxel-induced autophagy and apoptosis was further investigated using autophagy inhibitor 3-MA, which is a phosphoinositide 3-kinase inhibitor that exerts its autophagy-inhibiting effect before the formation of autophagosome[16]. As depicted in Fig. 4A, paclitaxel has increased the production of LC3-II in A549 cells, which was in accordance with the aforementioned results, however, pretreatment of cells with 3-MA (5 mmol/L) was effective in inhibiting the paclitaxel-induced conversion of LC3-I to LC3-II. Consistent with these results, cell viability inhibition and apoptosis induction by paclitaxel was markedly enhanced in the presence of 3-MA as shown in Fig. 4B and C. Collectively, these results suggest that inhibition of paclitaxel-induced autophagy sensitized the cells to paclitaxel with apoptosis induction.

3.5. Inhibition of autophagy by beclin 1 siRNA increases the cytotoxic sensitivity of cells to paclitaxel

The role of autophagy in the paclitaxel-mediated cytotoxicity was further studied by knocking down the beclin 1 expression using siRNA. Beclin 1 is a component of class III phosphatidylinositol 3-kinase complex essential for autophagosome formation[17]. The expression of beclin 1 was markedly suppressed in A549 cells transfected with beclin 1 siRNA but not those with random siRNA (Fig. 4A). Accordingly, cells transfected with beclin 1 siRNA showed reduced level of LC3-II accumulation after paclitaxel treatment when compared with random control (Fig. 4A). Consistent with the results using 3-MA, the cytotoxic effect of paclitaxel was significantly increased by blocking beclin 1 expression when compared to the random control (Fig. 4A). Similarly, the degree of apoptosis induced by paclitaxel was also enhanced when Beclin 1 protein level was reduced by the specific siRNA (Fig. 5C). As summarized in Fig. 5D, quantification of apoptotic cell shows that the incidence of apoptosis upon paclitaxel treatment was also higher in beclin 1 siRNA-transfected cells when compared to random siRNA control. These results demonstrate that autophagy induced by paclitaxel conferred protection of tumor cells against apoptosis, and blockade of autophagy subsequently enhanced the apoptosis associated with paclitaxel treatment.

3.6. Paclitaxel induces autophagy in other cancer cell lines

Furthermore, we extended our studies to test whether the autophagy-induction effect of paclitaxel is cell-type specific. As shown in Fig. 6A, after exposure 9 h, paclitaxel treatment led to the formation of GFP-LC3 punctate dots in human glioma cells U87, human prostate cancer PC-3 and colon cancer HT-29 cells which transfected with an expression plasmid containing GFP-LC3. These signals were evident in paclitaxel-treated cells when compared with the vehicle-treated control cells. In addition, the paclitaxel-induced production of LC3-II, Beclin 1 and Atg5 was also increased in U87, PC-3 and HT-29 cells as shown in Fig. 6B.

In addition, the role of autophagy in the paclitaxel-mediated cytotoxicity was also investigated using autophagy inhibitor 3-MA. The cell death after exposure of U87, PC-3 and HT-29 cells to various concentrations of paclitaxel for 24 h was significantly enhanced in the presence of 3-MA.
Taken together, these results suggested that the autophagy-activation effect of paclitaxel was unlikely to be cell-type specific, and inhibition of paclitaxel-mediated autophagy sensitized these cancer cells to paclitaxel.

4. Discussion

Despite having diverse mechanisms of action, many frontline anticancer agents would be predicted to stimulate autophagy including arsenic trioxide, 5-FU, histone deacetylase (HDAC) inhibitors, tamoxifen, imatinib, and ionizing radiation [18–23]. Paclitaxel is a widely used agent that is effective in the treatment of a variety of human cancers. Despite its capability to stabilize microtubules and impair mitosis, paclitaxel also exhibits induction of apoptosis, and regulation of cytokine gene expressions, suggesting the multiple effects of paclitaxel on human cancers [24,25]. In the present study, we have demonstrated that both autophagy and apoptosis are induced in cancer cells during the course of paclitaxel treatment.

Our results have shown that autophagic form of LC3-II was significantly increased after paclitaxel treatment in different cancer cell types including A549, U87, PC-3, and HT-29 cells. Meanwhile, we have also provided evidence that paclitaxel was able to upregulate several important autophagosome-regulatory genes such as Atg5 and beclin 1, and increase cytoplasmic formation of characteristic AVOs. All these results indicated that autophagy was induced by paclitaxel.

Prior studies have led to conflicting views of the role of autophagy in cancer chemotherapy. There is emerging evidence that autophagy plays critical roles in the generation of antineoplastic responses and mediates caspase-independent malignant cell death [26–28]. It has been established that autophagy mediates cell death of acute lymphoblastic leukemia cells by dexamethasone [29], promotes growth inhibition of PC3 cells by phenethyl isothiocyanate [30], promotes cell death by HDAC inhibitors in chondrosarcoma cell lines [31], and may constitute a
key mechanism by which TGFβ promotes generation of antitumor responses [32]. On the other hand, autophagy represents a protective mechanism against apoptotic cell death under starvation as well as contributes to resistance against therapy-induced apoptosis in cancer cells. It has been shown that autophagy is activated as a protective mechanism against 5-FU-induced apoptosis [33], autophagy blockade sensitizes prostate cancer cells towards sulforaphane [34], autophagy serves a protective role in imatinib mediated cell killing [21], and autophagy inhibition augments the anticancer activity of the histone deacetylase inhibitor SAHA [19]. In our study, we proceeded to dissect the contributions of autophagy to the survival of A549 cells during paclitaxel-induced apoptosis using autophagy inhibitor 3-MA. As expected, 3-MA inhibited paclitaxel-induced autophagy and enhanced paclitaxel-induced cell apoptosis in A549 cells. To further clarify the role of autophagy in paclitaxel-induced cell death, we used beclin1 siRNA to knock down beclin1 and evaluated the role of autophagy more directly. Consistent with the results using 3-MA, transfection of beclin1 siRNA effectively inhibited autophagosome formation and enhanced the cytotoxicity induced by paclitaxel in A549 cells. These findings suggest that paclitaxel-induced autophagy might provide a self-defense mechanism for cancer cells and inhibition of autophagy may enhance the therapeutic efficacy of paclitaxel in the treatment of cancer.

In conclusion, our data indicated that autophagy was induced in cancer cells along with paclitaxel-induced apoptosis, and inhibition of autophagy could result in potentiation of the proapoptotic effect of paclitaxel. Our findings suggest that appropriate modulation of autophagy is necessary for sensitizing tumor cells to anticancer therapy.

Conflicts of interest

None of the authors has any financial or other interest with regard to the submitted manuscript that might be constructed as a conflict of interest.

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