Cytoprotective role of autophagy during paclitaxel-induced apoptosis in Saos-2 osteosarcoma cells

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Abstract. Osteosarcoma (OS) is the most common primary malignant bone cancer in children and adolescents. Although paclitaxel (PCX) has been considered one of the most important cancer chemotherapeutic drugs, the current protocols for OS treatment do not incorporate this agent. Therefore, the purpose of this study was to evaluate the induction of cell death in OS cells after exposure to PCX, to identify the cell death mechanism(s) activated by PCX and to investigate whether autophagy is associated with PCX-induced apoptosis. The results of the present study confirmed that exposure to low PCX concentrations can induce apoptotic cell death in Saos-2 cells; furthermore, caspase-3 activation, PARP degradation and XIAP downregulation were observed in combination with PCX-induced apoptosis. The potential involvement of mitochondrial events (intrinsic apoptotic pathway) in PCX-induced apoptosis in OS cells was verified by the alteration (depolarization) of mitochondrial membrane potential. In addition, pretreatment with 3-methyladenine (3-MA), a specific inhibitor of autophagy, significantly increased PCX-induced apoptotic cell death in Saos-2 cells. The augmentation of PCX-induced apoptosis by 3-MA was accompanied by increase in the cytochrome c release from the mitochondria, caspase-3 activity and XIAP downregulation, which suggests that inhibiting autophagy further stimulates the PCX-induced mitochondrial cell death in Saos-2 cells. The activation of PCX-induced apoptosis by 3-MA was accompanied by increase in the cytochrome c release from the mitochondria, caspase-3 activity and XIAP downregulation, which suggests that inhibiting autophagy further stimulates the PCX-induced mitochondrial cell death in Saos-2 cells. The augmentation of PCX-induced apoptosis by 3-MA was accompanied by increase in the cytochrome c release from the mitochondria, caspase-3 activity and XIAP downregulation, which suggests that inhibiting autophagy further stimulates the PCX-induced mitochondrial cell death in Saos-2 cells.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone cancer in children and adolescents and is typically observed in individuals between the ages of 10 and 25 years (average age, 18 years) (1). In most cases, OS originates from the metaphysis of long bones and is mostly found in areas of rapid growth in children, i.e., the knees and shoulders and the long bones of the arms and legs (2). OS is highly aggressive and primarily metastasizes to the lungs (3). The 10-year disease-free survival rate is ~60 and 30% in patients with localized disease and patients with metastasis at diagnosis, respectively, with the current use of adjuvant and neoadjuvant chemotherapy involving doxorubicin, methotrexate, cisplatin and vincristine (4,5). Therefore, new therapeutic strategies need to be evaluated to improve OS survival, especially for patients refractory to current chemotherapy regimens.

Various chemotherapeutic drugs are reported to induce apoptosis in OS cells (6-8). Two apoptotic pathways have been proposed: one pathway involves cell death receptors (TNF-R or Fas) in the cell membrane, where binding of ligands to the receptors activates the caspase-8 and, in turn, activates downstream effector caspses (caspase-3 and -7) (9). The other pathway is associated with mitochondrial alterations such as decrease in mitochondrial membrane potential and release of cytochrome c from the mitochondrial membrane, followed by activation of effector caspses via the activation of caspase-9 (10). In both pathways, the activation of effector caspses is known to be suppressed by X-linked inhibitor-of-apoptosis protein (XIAP) (11). During the apoptotic process, the apoptotic mechanism can be classified as a caspase-dependent or caspase-independent pathway, depending on the involvement of caspase activation (12). The release of apoptosis-inducing factor (AIF) from the mitochondrial membrane is believed to be a specific marker for the caspase-independent pathway (13).
AIF contains a nuclear localization signal and translocates to the nucleus where it participates in chromatin condensation and large-scale DNA fragmentation (13).

During the last decade, autophagy has been gradually recognized by another type of cell death machinery in several cellular systems (14,15). Autophagy is a cellular process whose primary function is to degrade long-lived proteins and recycle cellular components (16). Autophagy can be induced by various stimuli, including starvation (17), cytokines (18), caspase inhibition (19) and chemical reagents such as rapamycin (20). In mammalian cells, autophagy has been implicated in cellular processes as varied as cell survival (17), death (19,20), pathogen clearance (18) and antigen presentation (21) and has also been associated with pathological processes such as cancer progression and neurodegenerative diseases (22,23).

Paclitaxel (Taxol®), a polyoxygenated naturally occurring diterpenoid isolated from the bark of the Pacific yew tree (Taxus brevifolia), has been considered one of the most important cancer chemotherapeutic drugs (24). PCX has a potent ability to stabilize microtubules. The anticancer activity of this drug is ascribed to its unique mechanism of action, i.e., causing mitotic arrest in cancer cells, which leads to apoptosis through inhibition of microtubule depolymerization (25). However, the current protocols for OS treatment do not incorporate this agent (26). The purpose of this study was to evaluate the induction of cell death in OS cells after exposure to PCX, to identify the cell death mechanism(s) activated by PCX and to investigate whether autophagy is associated with PCX-induced apoptosis.

Materials and methods

Reagents and antibodies. Paclitaxel (PCX) was obtained from Bristol-Myers Squibb (New York, NY, USA). 3-methyladenine (3-MA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and Rhodamine 123 (St. Louis, MO, USA). Antibody for microtubule associated protein 1 light chain 3 (LC3) was purchased from Novus Biologicals (Littleton, CO, USA). Antibodies for cytochrome c, β-actin was performed on nitrocellulose membrane. The membranes were transferred onto a nitrocellulose membrane. The membranes were incubated for 1 h at room temperature (RT) with a primary antibody in Tris-buffered saline containing 0.05% Tween-20 [TBS-T (pH 7.4)] in the presence of 5% non-fat dry milk. After the membranes were washed in TBS-T, secondary antibody reactions were performed with an appropriate source of antibody conjugated with horseradish peroxidase. The signals were detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) in the LAS-3000 detector (Fujifilm, Japan). Immunoblotting for β-actin was performed in each experiment as an internal control.

Preparation of mitochondrial fractions. The cells (5x10⁵) were washed in TD buffer (135 mM NaCl, 5 mM KCl, 25 mM Tris-Cl, pH 7.6) and allowed to swell for 15 min in ice-cold hypotonic CaRSB buffer [10 mM NaCl, 1.5 mM CaCl₂, 10 mM Tris-Cl (pH 7.5) protease inhibitors]. Cells were Dounce-homogenized with 30 strokes and mitochondria stabilization buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris, pH 7.6) was added. After removing nuclear contaminants (690 x g for 15 min), the supernatant was centrifuged at 20,800 x g for 15 min. The pellet (mitochondria) was directly diluted with lysis buffer [5 mM NaCl, 1 M Tris-Cl (pH 7.6), 5% Triton X-100, protease inhibitors] and the mitochondria (pellet) and supernatant (cytosol) were applied for protein analysis.

Caspase-3 activity assay. A fluorometric assay kit (Clontech, CA, USA), which contains fluorogenic substrate specific for caspase-3 immobilized in the wells, was used to evaluate enzyme activity. Ten micrograms of the extracted proteins in homogenization buffer (50 mM Tris-HCl, 150 mM NaCl,
10% glycerin and 1% Triton X-100) were added to the wells. The plate was incubated in the fluorescence plate reader at 37°C for 3 h and fluorescence was read every 10 min. The activity was determined by fluorometric detection (excitation, 380 nm; emission, 460 nm) and the negative control (blank, without sample) was subtracted from all the samples. Results at 2 h were selected, as the manufacturer suggested. Baseline values of negative controls and samples with specific inhibitors did not increase during the 2-h interval.

Measurement of mitochondrial membrane potential (MMP). The cells (5x10⁵) were incubated with 1 µM JC-1 dye at 37°C for 15 min, washed and resuspended with PBS and then the fluorescence [red (585/590 nm); green (510/527 nm)] was measured by flow cytometer.

Immunocytochemistry. Harvested cells were attached on the slide glass by cytospin centrifugation. The cells were fixed with 4% PFA, washed with PBS and incubated with 0.2% Triton X-100. Then, the cells were incubated with the appropriate primary antibody in 1% bovine serum albumin at RT. For secondary antibody reaction, the cells were incubated with an appropriate fluorescence-conjugated secondary antibody at RT. For counterstaining of the nucleus, if required, cells were incubated with PI (50 µg/ml) at RT. Finally, cells were mounted and observed under a confocal microscope (LSM510, Carl Zeiss, Germany).

Statistical analyses. Data were expressed as the mean ± SD of three or four separate experiments. Where appropriate, data were subjected analysis of variance (ANOVA) followed by Duncan’s post hoc test. Means were considered significantly different at p<0.05.

Results

PCX can induce cell death in osteosarcoma Saos-2 cells. To investigate whether PCX has a cytotoxic effect on osteosarcoma Saos-2 cells, the cells were exposed to various concentrations of PCX (2.5-100 nM) for up to 96 h. Cell viability decreased markedly by 2.5-5 nM PCX (Fig. 1A). The dose-response experiments showed that 5 nM of PCX is considered to be optimal for the time-course experiments in this study.

PCX-induced cell death exhibits apoptotic characteristics in Saos-2 cells. PCX-induced Saos-2 cell death was characterized by flow cytometry (Fig. 1B). Cell death was increased significantly in a dose-response manner (Fig. 3B), which confirmed that 5 nM of PCX is adequate for the time-course experiments. Annexin V staining was used to identify the type of cell death evoked by PCX (Fig. 1C). PCX significantly increased proportion of apoptotic cells in Saos-2 cells (Fig. 1C).

Caspase-3 activation and decrease of mitochondrial membrane potential are involved in PCX-induced apoptosis in Saos-2 cells. The involvement of caspase-3 activation in PCX-induced apoptosis was examined in Saos-2 cells. Exposure to PCX provoked an increase of caspase-3 cleavage (activation), which reached a post-exposure maximum level with 10 nM concentration at 48 h (Fig. 2A). Caspase-3 activation was accompanied not only by decrease in XIAP protein level but also by degradation of PARP protein (Fig. 2A). In addition, it has been observed that caspase-3 activities gradually increase during the duration of PCX treatment (Fig. 2B). In the present study, a decrease of MMP was observed in PCX-treated cells. The rate of mitochondrial depolarization showed a significant increase in PCX-treated cells compared to control (Fig. 2C).

Enhancement of PCX-induced apoptosis by the pretreatment of an autophagy inhibiting molecule. In order to investigate whether autophagic cell death is associated with PCX-induced
3-MA enhances cytochrome c release from mitochondria in PCX-treated cells. In order to examine whether inhibition of autophagosome formation correlates with the release of mitochondrial apoptotic proteins, the levels of these proteins were monitored in the cytosolic as well as mitochondrial fractions. We showed that 3-MA enhanced PCX-induced release of cytochrome c from the mitochondria (Fig. 5). In contrast, AIF was not released from the mitochondria under these conditions (Fig. 5). Furthermore, the levels of Bcl-2, an anti-apoptotic mitochondrial protein, appeared to be decreased after exposure to PCX, but were recovered in the presence of 3-MA (Fig. 5).

Discussion

During the last few decades, taxanes have been effectively used for the chemotherapeutic treatment of several types of cancers, including breast (27), lung (28), prostate (29) and ovarian (30) cancers. In addition, many in vitro experiments have shown that exposure to taxanes can induce apoptotic cell death in various types of cancer cells (31-33). PCX induces apoptosis in the human osteogenic sarcoma cell lines Saos-2 (34) and U-2 OS (35). Insufficient information is available on taxane-induced apoptosis in OS cells and therefore, taxanes have not been considered for chemotherapeutic regimens for treating OS (26).

Although previous studies have shown PCX-induced apoptosis and related mechanisms in OS cells (34,35), the concentrations of PCX (100-50,000 nM) used for inducing apoptosis were relatively higher than that used in the present study (5 nM). Because high-dose chemotherapy generally causes serious cytotoxic adverse effects in patients, it is important to develop lower-dose chemotherapeutic regimens. The results of the present study confirmed that exposure to low PCX concentrations can induce apoptotic cell death in Saos-2 cells; furthermore, caspase-3 activation, PARP degradation and XIAP downregulation were observed in combination with PCX-induced apoptosis. Both degradation of PARP and decrease in XIAP protein content appeared to be correlated with increased caspase-3 activity. In addition, the potential involvement of mitochondria (intrinsic apoptotic pathway) in PCX-induced apoptosis of OS cells was confirmed by the alteration (depolarization) of the mitochondrial membrane potential as well as the release of cytochrome c from the
Figure 3. Augmentation of paclitaxel-induced apoptosis in Saos-2 osteosarcoma cells by inhibition of autophagy. (A) Cells were treated with 5 nM PCX up to 96 h in the absence or presence of a specific autophagy inhibitor, 3-methyladenine (3-MA, 1 mM). The percentage of cells with a sub-G1 DNA content was taken as a measure of cell death. At least three independent experiments were performed and data shown are the mean ± SD. *p<0.05 compared to control cells. **p<0.05 compared to PCX-treated cells. ***p<0.001 compared to control cells. (B) Increase of caspase-3 activation and alterations of XIAP protein levels in Saos-2 osteosarcoma cells after exposure to paclitaxel (PCX) in the absence or presence of 3-methyladenine (3-MA, 1 mM). Western blot analysis of cleaved (active form) caspase-3 and XIAP at 48, 72 and 96 h after exposure to PCX (5 nM) treatment. Equal amounts of protein (30 µg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. NS, non-specific binding. Actin expression was used as a loading control. (C) Activation of caspase-3 and expression of LC3 proteins in the absence or presence of the pancaspase inhibitor z-VAD-fmk and/or 3-MA. Western blot analysis of cleaved (active form) caspase-3 and LC3 at 96 h after exposure to PCX (5 nM) treatment. Equal amounts of protein (30 µg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

Figure 4. Immunocytochemical localization of active caspase-3 and LC3 proteins in Saos-2 osteosarcoma cells after exposure to paclitaxel (PCX) in the absence or presence of 3-methyladenine (3-MA). The cells were treated with PCX (5 nM) for 96 h in the absence or in the presence of 3-MA (1 mM), cytopun, fixed and immunostained with the indicated antibodies. Green fluorescence (FITC) indicates immunoreactivity for active caspase-3 and LC-3. For differential nuclear staining, propidium iodide (PI, red) dye was employed. CON, control; PCX, paclitaxel-treated; PCX+3-MA, paclitaxel-treated cells in the presence of 3-MA. Microimages were taken using confocal microscopy. Original magnification, x400.
mitochondria to the cytoplasm. These results indicate that PCX-induced apoptosis in OS cells is associated with the caspase-dependent pathway, which is consistent with the previous findings for OS cells (34,35).

Similarly, involvement of caspase activation in taxane-induced apoptosis has been shown in cancer cells originating from the breasts (36), lungs (37), ovaries (38) and prostate (33). In contrast, several studies have shown that PCX-induced apoptosis is not related to caspase activity in some types of cancer cells (39,40). Currently, the precise reason for this inconsistency is not known, but the inconsistency is presumed to be due to cellular differences in tissue origin, status of differentiation, or cell cycle checkpoint/regulatory proteins such as p53 (41). The XIAP protein is an endogenous inhibitor of caspase-3 within cells (11) and has been suggested to be associated with chemoresistance in cancer cells (42). Recently, the potential role of XIAP in chemosensitivity or chemoresistance has been implicated in OS cells (43). However, XIAP downregulation in response to taxane therapy has not been shown in OS cells before this study. Therefore, developing a taxane chemotherapeutic regimen for XIAP downregulation could aid in inducing caspase-dependent apoptosis in OS cells.

Autophagy is currently considered as cell death machinery (programmed cell death type II) that differs from apoptosis (programmed cell death type I) (14). Dysregulation or malfunction of autophagy has been implicated in cancer (44), aging (45), diabetes (46), cardiovascular disease (47), inflammation (48) and neurodegenerative disease (49). Therefore, the selectivity of autophagy and its role in cell death and survival constitute an important issue for understanding the wide spectrum of human health and diseases (50). In the present study, pretreatment with 3-methyladenine (3-MA), a specific inhibitor of autophagy (51), significantly increased PCX-induced apoptotic cell death in Soas-2 cells. The augmentation of PCX-induced apoptosis by 3-MA was accompanied by increase in the cytochrome c release from the mitochondria, caspase-3 activity and XIAP downregulation, which suggests that inhibiting autophagy further stimulates the PCX-induced mitochondrial-related (intrinsic) apoptotic pathway by provoking caspase-3 activation.

Results similar to those obtained in this study have recently been reported for A549 lung cancer cells (52). In these studies, the anti-apoptotic function of autophagy was observed in PCX-induced apoptosis. Collectively, these results indicate the cytoprotective role of autophagy against apoptotic cell death in cancer cells. This cytoprotection does not seem to be restricted to taxane treatment in cancer cells. The cytoprotective function of autophagy has been observed during 5-fluorouracil treatment in colon cancer cells (53), sulforaphane treatment in prostate cancer cells (54), imatinib treatment in glioma cells (55) and suberoylanilide hydroxamic acid treatment in leukemia cells (56). Although the major cellular switch that determines cell destiny (cell death vs. cell survival) by autophagy after exposure to chemotherapeutic drugs remains unclear, the bcl-2 family proteins (bcl-2 and bcl-xl) in the mitochondria are believed to play a pivotal role in regulating autophagy (57).

Microtubule-associated protein light chain 3 (LC3) is now widely used to monitor autophagy (58). LC3 is modified via a ubiquitin-like system (59). LC3 exists in 2 forms: an 18-kDa cytosolic protein (LC3-I) and a processed 16-kDa form (LC3-II) in cells engaged in autophagy. The LC3-II form is mainly localized in autophagosome membranes and therefore, the LC3-II level is a good early marker for autophagosome formation (60). In the present study, immunoreactivity for LC3 markedly increased in Saos-2 cells upon PCX treatment but decreased in the presence of 3-MA. This obviously indicates that PCX treatment increased autophagy. It can be presumed that increased autophagy occurs during the rescue process of cellular structures (i.e., organelles) damaged by PCX exposure. Taken together, autophagy observed during PCX-induced apoptosis in Saos-2 OS cells represents the role of cytoprotection in cellular homeostatic processes.

In conclusion, the results of this study revealed that PCX exposure effectively induces OS cell death by apoptosis associated with the mitochondrial-mediated caspase-dependent pathway. PCX can increase autophagic activity and suppressing autophagy enhances PCX-induced apoptosis in OS cells. Therefore, it is suggested that combination treatment involving low-dose PCX therapy and autophagy inhibitor therapy could be an effective and potent strategy for improved chemotherapy for OS in the near future.