Inhibition of autophagy enhances cisplatin-induced apoptosis in the MG63 human osteosarcoma cell line

ZHICAI ZHANG, ZENGWU SHAO, LIMING XIONG and SHUHUA YANG

Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, P.R. China

Received December 24, 2014; Accepted August 17, 2015

DOI: 10.3892/ol.2015.3692

Abstract. Autophagy is a significant catabolic process that allows the renewal of intracellular organelles, through which cells are able to maintain homeostasis. In addition, autophagy may be associated with the carcinogenesis of osteosarcoma (OS). Cisplatin (CDDP) is an alkylating agent that is commonly used as an anticancer therapy. However, the pathways underlying the effects of CDDP remain to be elucidated. The present study demonstrated that 3-methyladenine (3-MA), an inhibitor of autophagy, was able to increase the proliferation inhibition ratios of MG63 human OS cells when used in combination with CDDP. Furthermore, MG63 cells produced significantly more microtubule-associated protein light chain 3II (LC3II), a widely used marker for monitoring autophagy, following CDDP treatment. Treatment with 3-MA was observed to inhibit these changes. Similarly, MG63 cells co-treated with 3-MA and CDDP demonstrated increased sensitivity to CDDP-induced apoptosis, compared with those exposed to CDDP alone. The present study revealed variation in the expression of LC3II and caspase-3 activity following treatment with certain drugs. The results of the present study suggest that CDDP may be capable of inducing apoptosis and autophagy, and that autophagy may be able to inhibit apoptosis in MG63 cells. Therefore, downregulation of autophagy may increase the chemotherapeutic sensitivity of MG63 cells to CDDP.

Introduction

Osteosarcoma (OS) is the most commonly observed primary bone tumor in children (1). It is an aggressive malignant tumor, and >80% of patients treated with surgery alone exhibit distant metastases (2). Standard therapy is typically multimodal, consisting of neoadjuvant chemotherapy and subsequent amputation or limb-sparing reconstructive surgeries, in combination with adjuvant chemotherapy (3). CDDP, a common antitumor drug, has been found to be effective against cancer cells derived from solid tumors, such as osteosarcoma, hepatoma and thymoma. In clinical treatment, CDDP is a common choice for osteosarcoma chemotherapy (4).

Advances in molecular technologies have facilitated an increased understanding of the mechanisms underlying carcinogenesis, primarily focusing on the recognized model of multistage carcinogenesis with underlying progressive genetic changes, which induce malignant transformation (5). Recently, the mechanisms underlying autophagy have gained increasing attention.

Autophagy describes the bulk degradation of proteins and organelles, and is an essential process for effective cellular maintenance, viability, differentiation and mammalian development (6). In mammals, autophagy has been observed in numerous tissues, and has been demonstrated to possess significant associations with neurodegenerative diseases, cardiomyopathies, tumors and apoptosis, as well as bacterial and viral infections (7). It has been proposed that autophagy may be capable of protecting certain cancer cells from anticancer therapies by blocking apoptotic pathways, whereas other cancer cells are observed to undergo autophagic cell death following anticancer therapy (8). The opening of the mitochondrial permeability transition pore induces autophagy and apoptosis, and a small quantity of mitochondria spontaneously depolarize and induce autophagy (9). In this way, autophagy eliminates dysfunctional mitochondria, so that cells are protected from apoptosis.

Whilst several signaling pathways are implicated in autophagic cell death, the PI3K-AKT-mTOR signaling pathway appears to play a pivotal role. 3-MA, an inhibitor of class III PI3K, has been used to suppress autophagy (10). Microtubule-associated protein light chain 3II (LC3II) is increasingly being used to monitor levels of autophagy; the expression levels of LC3II have been observed to correlate with the number of autophagosomes, and therefore this is potentially a useful method (11).

In the present study, MG63 OS cell proliferation inhibition ratios were evaluated following the application of various drugs. In particular, the present study focused on apoptosis, the formation of autophagosomes under various conditions and the expression of characteristic autophagy genes, including LC3II and the apoptosis-activated protein caspase-3.
Materials and methods

Cell culture and experimental design. The MG63 human OS cell line was cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; both from HyClone, Logan, UT, USA), 1% penicillin (50 U/ml)/streptomycin (50 µg/ml) (Gibco Life Technologies, Carlsbad, CA, USA) and 5 µg/ml Plasmocin™ prophylactic (InvivoGen, San Diego, CA, USA) in a humidified atmosphere with 5% CO₂, in a water-jacketed incubator at 37°C. The MG63 cell line was obtained from the China Center for Typical Culture Collection (Wuhan University, Wuhan, Hubei, China).

CDDP and 3-MA were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG63 cells were divided into four groups: Control group, cisplatin (CDDP) group, 3-methyladenine (3-MA) group and CDDP + 3-MA group. Control group cells were treated with phosphate-buffered saline (PBS; Sigma-Aldrich). The CDDP group was exposed to CDDP (10 µg/ml), the 3-MA group was treated with 3-MA (6 mM) and the CDDP + 3-MA group was treated with CDDP (10 µg/ml) and 3-MA (6 mM). Cells were exposed to the drugs for 24 h.

MTT assay. MG63 cells were cultured until mid-log phase in order to obtain a stock cell suspension that contained 1x10⁵ cells/l. The stock cell suspension (100 µl) was subsequently added to a 96-well plate. Following 24 h of culture, cells were treated with the drugs, and subsequently incubated at 37°C in 5% CO₂ for 24 h. Following incubation, 20 µl MTT stock solution (5 mg/ml in PBS; Amresco LLC, Solon, OH, USA) was added to each well. Cells were subsequently incubated at 37°C for 4 h, then centrifuged at 1,000 x g for 10 min at 37°C. Supernatant was discarded, 150 µl dimethyl sulfoxide (Sigma-Aldrich) was added and the plate was agitated for 10 min in the dark. Finally, the optical density (OD) was detected using a microplate reader (µQuant™; Bio-Tek Instruments, Inc., Winooski, VT, USA) at a wavelength of 490 nm. The following formula was utilized in order to calculate cell proliferation inhibition ratios:

Inhibition ratio = \[ 1 - \left( \frac{OD_{\text{experimental sample}}}{OD_{\text{control}}} \right) \] x 100%.

Laser scanning confocal microscopy. Cells in the four groups were incubated in medium with various supplements, as indicated. Following 24 h of incubation, cells were plated on coverslips and fixed in 4% formaldehyde (Sigma-Aldrich) in PBS for 20 min at 4°C, rinsed in PBS and exposed to an incubation buffer (5% FBS in PBS) for an additional 20 min at room temperature. Cells were subsequently treated with 1:200 diluted polyclonal rabbit anti-human antibody to LC3II (sc-28266; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 12 h in a humidifier at 4°C. Following rinsing in the incubation buffer (twice for 5 min), the specimens were incubated with 1:100-diluted goat anti-rabbit IgG-FITC conjugate (Invitrogen Life Technologies, Carlsbad, CA, USA) and propidium iodide (PI; Sigma-Aldrich) for 1 h at room temperature, and subsequently rinsed with PBS. Fluorescence was detected using a laser confocal microscope (TCS SP2; Leica Microsystems GmbH, Wetzlar, Germany).

Flow cytometry. Following treatment for 24 h with various drugs, cells were collected by centrifugation at 1500 x g for 10 min at 4°C, fixed in 4% formaldehyde, rinsed with PBS and exposed to an incubation buffer at 4°C. Cells were treated with 1:200 diluted polyclonal rabbit anti-human antibody to LC3II for 12 h at 4°C. Following rinsing with the incubation buffer, specimens were exposed to 1:100 diluted goat anti-rabbit IgG-FITC conjugate for 1 h at room temperature and subsequently rinsed with PBS.

Apoptosis was quantified by combined staining with Annexin V and PI using an Annexin V-FITC Apoptosis Detection kit (MBL International Co., Woburn, MA, USA). Briefly, 24 h subsequent to treatment with various drugs, cells were collected by centrifugation at 1500 x g for 10 min at 4°C, and dissolved in 500 µl 1X binding buffer. Following the addition of 10 µl Annexin V-FITC solution and 5 µl PI solution, cells were incubated for 15 min at room temperature in the dark.

Following incubation, cells were analyzed using a flow cytometer (FACSort; BD Biosciences, Franklin Lakes, NJ, USA) equipped with an argon laser and filter configuration for FITC/PI dye combination. Light scattering and fluorescence signals were subjected to linear and logarithmic amplifications, respectively. At least 10,000 events were acquired and analyzed using Cell Quest software version 5.1 (BD Biosciences). All the experiments were performed at least three times for each condition. The data were plotted on a logarithmic scale.

Western blot analysis. Cytosolic fraction proteins were separately collected using an Apo Alert Cell Fractionation kit (Clontech Laboratories, Inc., Mountainview, CA, USA). MG63 cells were trypsinized (Sigma-Aldrich), collected, resuspended in ice-cold wash buffer and then centrifuged at 700 x g for 5 min at 4°C. Following removal of the supernatant, cells were resuspended in 200 µl ice-cold fractionation buffer mix and placed on ice for 10 min. The homogenates were centrifuged at 10,000 x g for 10 min at 4°C, and supernatants were transferred to a fresh tube. Supernatants were collected and used for Western blot analysis.
as a cytosolic fraction. Protein samples (30 mg) were electrophoresed on a 12.5% SDS gel (Clontech Laboratories, Inc.) and transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amresco, LLC, Solon, OH, USA). Membranes were blocked by incubation with 4% skim milk in PBS with Tween (PBST; Clontech Laboratories, Inc.) at 4˚C overnight. Following rinsing, membranes were incubated with rabbit anti-human polyclonal anti-LC3II and rabbit anti-human monoclonal anti-caspase-3 (#N791; Amresco) antibodies (1:1,000 dilution), respectively, overnight at 4˚C. Following five washes with PBST, membranes were incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody; Amresco) for 1 h at room temperature. Following rewashing with PBST (5 times), the membranes were analyzed using an ECL Western Blotting Substrate Kit (Amresco). X-ray films were scanned on a flat-bed scanner, and western blotting results were quantified using Photoshop Image Analysis software CS3 (Adobe Systems, Inc., San Jose, CA, USA).

Statistical analysis. SPSS 10.0 package (SPSS, Inc., Chicago, IL, USA) was utilized for statistical analysis. Data are expressed as a cytosolic fraction. Protein samples (30 mg) were electrophoresed on a 12.5% SDS gel (Clontech Laboratories, Inc.) and transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amresco, LLC, Solon, OH, USA). Membranes were blocked by incubation with 4% skim milk in PBS with Tween (PBST; Clontech Laboratories, Inc.) at 4˚C overnight. Following rinsing, membranes were incubated with rabbit anti-human polyclonal anti-LC3II and rabbit anti-human monoclonal anti-caspase-3 (#N791; Amresco) antibodies (1:1,000 dilution), respectively, overnight at 4˚C. Following five washes with PBST, membranes were incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody; Amresco) for 1 h at room temperature. Following rewashing with PBST (5 times), the membranes were analyzed using an ECL Western Blotting Substrate Kit (Amresco). X-ray films were scanned on a flat-bed scanner, and western blotting results were quantified using Photoshop Image Analysis software CS3 (Adobe Systems, Inc., San Jose, CA, USA).

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as the mean ± standard error. Single-factor analysis of variance was performed for each treatment group. P<0.05 was considered to indicate a statistically significant difference. Experiments were repeated at least three times for each condition.

Results

Inhibition of autophagy enhances CDDP-induced cell death.

A previous study revealed that CDDP promotes apoptosis in OC cells (12). To determine the cytotoxicity of CDDP and 3-MA on MG63 cells, the present study investigated the effects of these drugs on cell proliferation using an MTT assay. As illustrated in Fig. 1, CDDP significantly increased the percentage of Annexin V+/PI- (apoptotic cells) compared with that of control cells (P<0.05). 3-MA + CDDP co-treatment significantly increased the percentage of Annexin V+/PI- (apoptotic cells) compared with that of cells treated with CDDP alone (P<0.05). There was no significant difference between the untreated control and 3-MA groups (P>0.05). PI, propidium iodide; CDDP, cisplatin; 3-MA, 3-methyladenine; FITC, fluorescein isothiocyanate.

CDDP treatment induces autophagy in MG63 cells.

Autophagy is an alternative form of cell death to apoptosis, characterized by the generation of autophagic vesicles, and the degradation of cytoplasmic components and organelles (13). LC3II, a novel autophagosome marker, was utilized in the present study (14). In order to confirm the role of CDDP-induced autophagy, the present study investigated the expression of LC3II in MG63 cells using confocal microscopy. As illustrated in Fig. 2, the fluorescence intensity of LC3II was upregulated following CDDP treatment (P<0.05). There was no significant difference between the control and 3-MA groups (P>0.05). Data are expressed as the mean ± standard error. LC3II, microtubule-associated protein light chain 3II; CDDP, cisplatin; 3-MA, 3-methyladenine.

Figure 4. Flow cytometric analysis of MG63 cell apoptosis. (A) Representative plots of Annexin V vs. PI fluorescence for control cells and cells treated with CDDP, 3-MA and CDDP + 3-MA for 24 h. Q1, Annexin V-/PI-, damaged cells; Q2, Annexin V+/PI-, necrotic cells; Q3, Annexin V-/PI, viable cells; and Q4, Annexin V+/PI, apoptotic cells. (B) Percentage of cells labeled with Annexin V+/PI (apoptotic cells), Annexin V-/PI (damaged cells) and Annexin V-/PI (necrotic cells) following 24 h of treatment. Data are presented as the mean ± standard error. CDDP significantly increased the percentage of Annexin V+/PI (apoptotic cells) compared with that of control cells (P<0.05). 3-MA + CDDP co-treatment significantly increased the percentage of Annexin V+/PI (apoptotic cells) compared with that of cells treated with CDDP alone (P<0.05). There was no significant difference between the untreated control and 3-MA groups (P>0.05). PI, propidium iodide; CDDP, cisplatin; 3-MA, 3-methyladenine; FITC, fluorescein isothiocyanate.

Figure 5. LC3II and caspase-3 expression in MG63 cells. (A) Western blot analysis of LC3II and caspase-3 expression in the cell populations. (B) The expression of LC3II and caspase-3 proteins was increased following CDDP treatment (P<0.05), while combined CDDP + 3-MA treatment downregulated the expression of LC3II and upregulated the expression of caspase-3 (P<0.05). There was no significant difference between the control and 3-MA groups (P>0.05). Data are expressed as the mean ± standard error. LC3II, microtubule-associated protein light chain 3II; CDDP, cisplatin; 3-MA, 3-methyladenine.
Inhibition of autophagy increases CDDP-induced apoptosis of MG63 cells. To investigate the association between autophagy and apoptosis in MG63 cells treated with CDDP, MG63 cells were co-treated with CDDP and 3-MA and the degree of apoptosis was assessed. The percentage of cells undergoing apoptosis was quantified using flow cytometry. apoptotic (Annexin V+/PI) and necrotic (Annexin V−/PI−) cells were distinguished on the basis of double-labeling for Annexin V-FITC and PI (Fig. 4A). As indicated in Fig. 4B, untreated control MG63 cells demonstrated slight fluorescent staining. By contrast, treatment with CDDP alone for 24 h induced a significant increase in the levels of apoptosis in MG63 cells compared with control cells (P<0.05), and CDDP and 3-MA co-treatment significantly increased the percentage of apoptotic MG63 cells compared with that of cells treated with CDDP alone (P<0.05). Following 24 h of incubation, there was no significant increase in the number of necrotic (Annexin V+/PI+) and damaged (Annexin V−/PI−) cells between the untreated control group and the 3-MA-treated group. Thus, the results of the present study demonstrated that inhibition of autophagy by 3-MA significantly increased the apoptotic effects of CDDP in MG63 cells.

Differential effects of CDDP and 3-MA may be due to the expression of LC3II and caspase-3 in MG63 cells. The present study investigated the protein expression of LC3II and caspase-3 in MG63 cells. As illustrated in Fig. 5, western blot analyses revealed that the expression levels of LC3II and caspase-3 proteins were increased in MG63 cells following CDDP treatment. Furthermore, reduced levels of LC3II protein and increased levels of caspase-3 protein were detected in the CDDP + 3-MA co-treatment group, compared with those of the CDDP-treated group (P<0.05). The results of the present study suggest that the differential effects of CDDP and 3-MA on MG63 cells may be due to the expression of proapoptotic and autophagic proteins.

Discussion

The results of the present study revealed that autophagy protected MG63 human OC cells from CDDP-induced apoptosis, and inhibition of autophagy mediated by 3-MA enhanced the sensitivity of MG63 cells to the apoptosis inducer CDDP. CDDP is an alkylating agent that reacts with DNA and cellular proteins, and the magnitude of cell death is well correlated with tumor response to CDDP (15). The molecular mechanisms through which CDDP induces apoptosis are DNA crosslinking, inhibition of DNA replication and RNA transcription (16). In the present study, it was demonstrated that CDDP specifically induced apoptosis via caspase-3 activation in MG63 cells.

Although a number of researchers have hypothesized potential mechanisms, the signaling pathways of the target molecules of CDDP that lead to cell death have not yet been elucidated (17). Previous studies have revealed that numerous chemotherapeutic agents are capable of inducing autophagic cell death. The alkylating agent temozolomide is capable of killing malignant glioma cells via the process of autophagic cell death (18). Comparably, resveratrol is capable of inducing autophagic cell death in ovarian cancer cells (19).

Autophagy is characterized by the appearance of abundant cytoplasmic autophagic vacuoles, and an increase in the size of the endoplasmic reticulum and Golgi apparatus (20). The LC3II protein, located in the autophagosomal membrane, may be used as a general marker for the autophagic membrane (21). 3-MA is an inhibitor of the class III phosphatidylinositol 3-kinases and is known to be involved in the initial phase of autophagy (22). A previous study investigating 3-MA demonstrated that it was capable of increasing the sensitivity of HT-29 colon cancer cells to apoptosis induced by a cyclooxygenase inhibitor, sulindac sulfide (23). In the present study, 3-MA was used in combination with CDDP, and the results revealed that the sensitivity to chemotherapy may be increased by the downregulation of autophagy. The findings of the aforementioned studies suggest that autophagy may inhibit apoptosis by sequestering mitochondrial death-promoting factors, for example cytochrome c (24). However, the mechanism of autophagic activity, which promotes the protection of cells from apoptosis remains to be elucidated.

Recent advances in our understanding of the molecular mechanisms underlying anticancer therapy-induced autophagy and apoptosis, have provided significant information for studying tumor responses, in terms of the signal transduction pathways of cell death (25). This understanding potentially facilitates the design of targeted therapies for the promotion of cancer cell sensitivity to anticancer treatments.

References


