Autophagy is associated with apoptosis in cisplatin injury to renal tubular epithelial cells

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Yang C, Kaushal V, Shah SV, Kaushal GP. Autophagy is associated with apoptosis in cisplatin injury to renal tubular epithelial cells. Am J Physiol Renal Physiol 294: F777–F787, 2008. First published February 6, 2008; doi:10.1152/ajprenal.00590.2007.—Autophagy has emerged as another major “programmed” mechanism to control life and death much like “programmed cell death” is for apoptosis in eukaryotes. We examined the expression of autophagic proteins and formation of autophagosomes during progression of cisplatin injury to renal tubular epithelial cells (RTEC). Autophagy was detected as early as 2–4 h after cisplatin exposure as indicated by induction of LC3-I, conversion of LC3-I to LC3-II protein, and upregulation of Beclin 1 and Atg5, essential markers of autophagy. The appearance of cisplatin-induced punctuated staining of autophagosome-associated LC3-II upon GFP-LC3 transfection in RTEC provided further evidence for autophagy. The autophagy inhibitor 3-methyladenine blocked punctated staining of autophagosomes. The staining of normal cells with acridine orange displayed green fluorescence with cytoplasmic and nuclear components in normal cells but displayed considerable red fluorescence in cisplatin-treated cells, suggesting formation of numerous acidic autophagolysosomal vacuoles. Autophagy inhibitors LY294002 or 3-methyladenine or wortmannin inhibited the formation of autophagosomes but induced apoptosis after 2–4 h of cisplatin treatment as indicated by caspase-3/7 and -6 activation, nuclear fragmentation, and cell death. This switch from autophagy to apoptosis by autophagic inhibitors further suggests that the preapoptotic lag phase after treatment with cisplatin is mediated by autophagy. At later stages of cisplatin injury, apoptosis was also found to be associated with autophagy, as autophagic inhibitors and inactivation of autophagy proteins Beclin 1 and Atg5 enhanced activation of caspases and apoptosis. Our results demonstrate that induction of autophagy mounts an adaptive response, suppresses cisplatin-induced apoptosis, and prolongs survival of RTEC.

LC3; Beclin 1; ATG5; caspase-3; caspase-6; acute kidney injury

Autophagy regulates cell death in both physiological as well as pathophysiological conditions (21, 22). A low level of constitutive autophagy occurs under normal physiological conditions (12, 21, 29). While under normal conditions basal autophagy is a mechanism for the turnover of proteins and elimination of damaged or aged organelles and cytoplasmic components to maintain cell homeostasis, autophagy induction under pathological conditions is generally considered to provide a prosurvival role; however, extensive autophagy or inappropriate activation of autophagy results in cell death by bulk elimination of cells. Also known as type II programmed cell death, autophagic cell death has been reported during embryonic development and under pathophysiological conditions that require extensive autophagy for large-scale elimination of cells (11, 19, 21, 22, 25). Autophagic cell death is distinct from apoptosis (also termed type I programmed cell death) in that it is caspase independent and has been shown to occur in the presence of caspase inhibitors (37) and when the apoptosis machinery is defective (34). Under the condition of stress signals, induction of autophagy plays a role in cellular survival rather than cell death. In this process, the autophagy-mediated production of amino acids, fatty acids, sugars, and other essential metabolites produced from the degradative process are recycled (21, 22, 25) for energy production and protein synthesis that allows adaptation against cellular environmental changes. The survival function of autophagy is well established in nutrient- or growth factor-deprived cells for adaptation where degradative metabolites produced by autophagy are utilized for energy sources for cell survival (19, 21, 24). Under the conditions of cellular stress, the inhibition of autophagy has been shown to result in apoptosis (3, 4, 6). Cisplatin is known to cause cellular stress including oxidative stress and DNA damage (2, 8, 14); however, its role in the induction of the autophagic pathway in renal tubular epithelial cells (RTEC) has not been previously examined. It is not known whether autophagy is induced in renal injury and, if so, whether it plays a prosurvival or prodeath role.

Previous studies from our lab (16) and that of others (9, 23) demonstrated that, in response to cisplatin, activation of caspases and cell apoptosis did not begin until 8 h of cisplatin treatment and, thereafter, caspase activation and cell apoptosis occurred in a time- and dose-dependent manner. These studies suggested the occurrence of a preapoptotic lag phase of cell survival in response to cisplatin injury. We were interested to unravel the mechanism of cell survival for the initial 8 h after exposure of cisplatin to renal cells. Since recent studies re-
Figure A: Caspase-3/7, 6 Activity (p moles) over time course (hrs).

Figure B: Control, 4h CP, 8h CP, 12h CP, 18h CP, 24h CP

Figure C: Cell Death (%) over time course (hrs).

Figure D: Control, 2h CP, 4h CP, 8h CP, 12h CP, 24h CP
ported that, under stress conditions, autophagy is induced and plays an adaptive role for cell survival, we hypothesized that the cisplatin-induced lag phase of cell survival in renal cells may occur due to induction of the autophagic pathway. Thus, in the present study we determined that autophagy is associated in cisplatin injury with RTEC, involved in the cisplatin-induced adaptive response, and delays the cisplatin-induced apoptotic response. We examined the expression of essential proteins involved in the autophagic pathway and the effect of autophagy inhibition on cisplatin-induced cell death using a small interfering RNA approach and specific inhibitors of autophagosome formation during the progression of cisplatin injury to RTEC.

MATERIALS AND METHODS

Cell culture and reagents. LLC-PK1 cells obtained from ATCC were cultured as described in our previous studies (17). The cells were grown in Gibco medium 199 supplemented with 10% heat-inactivated fetal calf serum. Cultures were maintained in a humidified incubator gassed with 5% CO2-95% air at 37°C and fed with fresh medium at intervals of 48–72 h. Experiments were performed with cells grown to 70–80% confluence. Caspase substrates were purchased from Peptide International (Louisville, KY) and antibodies to proform (8G10, cat. no. 9665) and active forms (Aasp175, cat. no. 9661) of caspase-3 and caspase-6 (cat. no. 9762) were obtained from Cell Signaling Technology (Beverly, MA). Caspase substrates Asp-Glu-Val-Asp-aminomethyl coumarin (DEVD-AMC) for caspase-3/7, Val-Glu-Ile-Asp-aminomethyl coumarin (VEID-AMC), and Tyr-Val-Ala-Asp-aminomethyl coumarin (YVAD-AMC) were purchased from Peptide International. Caspase inhibitors, benzoyloxycarbonyl-Val-Ala-Asp-fluoro-methylethylketone (Z-VAD-fmk), benzoyloxycarbonyl-Val-Ala-Asp-Val-Ala-Asp-fluoromethylketone (Z-VDAD-fmk), and benzoyloxycarbonyl-Asp-Glu-Val-Ala-Asp-fluoromethylketone, were obtained from Enzyme System Products (Livermore, CA). Antibodies to α-actinin (cat. no. sc-17829) and α-tubulin (cat. no. sc-8035) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bafilomycin A1 and 3-methyladenine (3MA) were obtained from Enzyme System Products (Livermore, CA). Antibodies to phosphoinositol (PI) 3-kinase, and wortmannin, a potent inhibitor of PI 3-kinase, were obtained from Calbiochem (San Diego, CA).

Induction of cisplatin-induced injury. The cell culture medium was replaced with fresh medium containing serum, and cells were incubated either without or with cisplatin at various concentrations (25–200 μM) for the period of time indicated (1–24 h). In initial studies, we determined the optimum exposure time and the suitable concentration of cisplatin. To determine the effect of inhibitors, cells were treated with the inhibitors for 10 min before the addition of cisplatin (50 μM).

Caspase activity assay. Cells were harvested by centrifugation, and the pellets were washed in cold PBS twice. The washed cell pellets were lysed with 20 mM HEPES, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A at 4°C. The supernatants obtained were used to determine the activities of caspase-3/7, -6, and -1 by a fluorometric assay using the following amino-4-methylcoumarin (AMC)-tagged substrates: DEVD-AMC for caspase-3/7, VEID-AMC for caspase-6, and YVAD-AMC for caspase-1, as described previously (16). The cell extracts containing 50 μg of protein were incubated with 100 mM HEPES, pH 7.4, containing 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, and 50 μM caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 60 min at 30°C. At the end of the incubation, the amount of liberated fluorescent group, AMC, the data for caspase activity are expressed as nanomoles of AMC liberated when 50 μg of protein extract are incubated with 50 μM substrate for 60 min at 30°C.

GFP-LC3 overexpression and autophagy detection. LLC-PK1 cells were plated at a density of 2 × 10^4 on glass coverslips in six-well plates and cultured up to 70% confluence. Cells were transfected with GFP-LC3 plasmid DNA (kindly provided by Dr. T. Yoshimori, Osaka University, Japan) for 36 h and then treated with 50 μM cisplatin for various time points. Transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer’s recommendation and 2 μg/ml GFP/LC3 plasmid DNA in each well was used. Microphotographs of GFP-LC3 fluorescence were obtained with a fluorescence microscope. The detection of punctuated staining of GFP-LC3 from the diffuse staining indicated the formation of autophagosomes.

Detection of autophagic vacuoles by acridine orange. LLC-PK1 cells were seeded in T-25 flasks and at 70% confluence cells were untreated or treated with 50 μM cisplatin for various time points. At the appropriate time points, cells were incubated with 1 μg/ml acridine orange (Molecular Probes, Eugene, OR) in serum-free medium for 15 min. The acridine orange was removed and fluorescent micrographs were obtained using an inverted fluorescence microscope. The cytoplasm and nucleus of the stained cells fluoresced bright green, whereas the acidic autophagic vacuoles fluoresced bright...
red. Cells were treated with 200 nmol/l bafilomycin A1 for 30 min before the addition of acridine orange to inhibit the acidification of autophagic vacuoles.

**Western blot analysis.** The cell lysates were prepared as described above for the caspase assay, and equal amounts of protein samples were resolved by SDS-polyacrylamide gel electrophoresis using Novex NuPAGE 4–12% Bis-Tris polyacrylamide gels (Invitrogen). The proteins were electrophoretically transferred to a Trans-Blot membrane (Bio-Rad), processed for immunoblotting with specific antibodies, and detected using the ECL system as previously described (16).

**RNA interference for Beclin 1 and Atg5.** LLC-PK1 cells were plated in a six-well plate with complete medium. When the cells were 70% confluent, the old medium was replaced with fresh medium without serum and antibiotics. Beclin siRNA (sc-29798) and Atg5 siRNA (sc-41446) were obtained from Santa Cruz Biotechnology. Cells growing at 70% confluence were transfected according to the manufacturer’s instructions. Briefly, 8 μl siRNA transfection reagent and 80 pmol siRNA in 100 μl siRNA transfection media were mixed and incubated at room temperature for 20 min. The cells in each well were then transfected with this mixture. After 12 h, fetal bovine serum was added to a final concentration of 10%, and on day 2, the medium in the cells was replaced with fresh medium before cisplatin treatment.

**Cell viability and cell apoptosis.** Inhibition of cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Roche Molecular Biochemicals, Laval, PQ, Canada) according to the manufacturer’s protocol. To determine whether cell death was through apoptosis, cells were grown on glass coverslips in six-well plates. The cells were treated with autophagic inhibitors 30 min before treatment with 50 μM cisplatin at various times. Apoptosis was detected by the TUNEL assay using a TdT-FragEL DNA fragmentation detection kit (cat. no. QIA33, Calbiochem, La Jolla, CA).

![Fig. 3. Fluorescence staining of GFP-LC3 in response to cisplatin injury. LLC-PK1 cells were plated at 70% confluent on glass coverslips. Following transfection with GFP-LC3 plasmid as described in MATERIALS AND METHODS, cells were treated with cisplatin for various times as indicated, fixed with paraformaldehyde, and observed using a single-photon confocal microscope. The appearance of cisplatin-induced punctated staining indicates autophagosome-associated LC3-II. The autophagic inhibitor 3-methyladenine (3MA) was used to examine the effect in cisplatin-induced punctated staining.](image-url)
following the manufacturer’s instructions. Apoptosis was also detected using DAPI (1.5 μg/ml) staining. Images were viewed and captured using an Olympus microscope equipped with a digital camera, model DP71. The apoptotic cells were counted in five fields and compared by two-tailed, unpaired t-tests between two groups.

Statistical analysis. An ANOVA test and Student’s t-test were used to compare mean levels. P < 0.05 was considered statistically significant.

RESULTS

Caspase activation and apoptosis in cisplatin injury. We previously reported significant activation of caspase-3 following cisplatin exposure to RTEC but this activation did not occur until 8 h of cisplatin treatment (16). The initiation of caspase-3 activation after 8 h of cisplatin treatment was also accompanied by activation of other executioner caspases, caspase-6 and caspase-7 (Fig. 1A). In these studies, the activities of caspase-3 and -7 were determined together because caspase-3 and -7 utilize the same substrate, DEVD-AMC; therefore, the activities determined by cleavage of DEVD-AMC are in fact contributed by both of these caspases. The cisplatin-induced lag phase in activation of caspase-3/7 and -6 was also reflected in the absence of apoptosis during this period as determined by DAPI staining (Fig. 1, A and C) and the TUNEL assay (Fig. 1D). Since, under the condition of cellular stress, the induction of autophagy manifests a survival role, we hypothesized that a cisplatin-induced preapoptotic lag phase of cell survival may occur due to induction of autophagy.

Induction of autophagy and its detection in cisplatin injury. One of the established steps in autophagy during autophagosome formation is the induction of microtubule-associated protein 1 light chain 3 (LC3) and its conjugation with phosphatidylethanolamine (PE) (13, 27, 35). The cytosolic 18-kDa LC3 (also termed LC3-I) form is converted to the autophagosome-associated 16-kDa LC3-II form (faster migrating on SDS gels) by the lipid PE conjugation. The conversion of LC3-I to LC3-II is considered a reliable marker of autophagy (15, 27).

We examined whether LC3-I is converted to LC3-II during cisplatin injury to renal cells. Western blot analysis indicated not only induction of LC3-I but also conversion of LC3-I to LC3-II in response to cisplatin exposure. This conversion began very early at 2 h of cisplatin treatment and gradually increased until 8 h; this increase became steady at the later periods of cisplatin treatment (Fig. 2). The recruitment of LC3-II to autophagic vesicles was further tested by expression of GFP-LC3 in LLC-PK1 cells in response to cisplatin treatment. In transfected cells, the punctate accumulation of GFP-LC3-II, characteristic of autophagy (15), was detected before the onset of apoptosis, and many cells showed punctate staining at 4 h and later periods following cisplatin exposure (Fig. 3).

Control
CP 4h
CP 8h
CP 16h
CP 24h

Beclin
Actin
Atg5

Fig. 4. Induction of autophagic proteins Beclin 1 and Atg5 in response to cisplatin. LLC-PK1 cells were treated with 50 μmol/l for various time periods as indicated. Beclin 1 and Atg5 induction and expression were determined by Western blot using an antibody specific to Beclin 1 and Atg5.

During autophagy, acidic autophagic vacuoles, also called autophagosomes, are formed as a result of fusion of autophagosomes with lysosomes (6, 31) and are considered a characteristic feature of cells engaged in autophagy. Formation of autophagosomes can be detected by fluorescence microscopy following staining with the lysosomotropic agent acridine orange. Staining of normal cells with acridine orange, a weak base, displays green fluorescence with cytoplasmic and nuclear components. In larger acidic compartments such as autophagolysosomes, the protonated form of acridine orange accumulates and displays red fluorescence when observed by fluorescence microscopy. We therefore determined the effect of cisplatin on the formation of autophagolysosomes by fluorescence microscopy following staining with acridine orange. Untreated control cells showed predominantly green fluorescence with very minimal red fluorescence, whereas cells treated with cisplatin displayed considerable red fluorescence (Fig. 5), both at the preapoptotic lag phase as well as in later periods of cisplatin treatment, suggesting formation of numerous autophagolysosomal vacuoles during the course of cisplatin injury. Bafilomycin A1 is an inhibitor of autophagy that acts by inhibiting the H⁺-ATPase responsible for acidification of the autophagolysosomal vacuoles (38). Preincubation of cisplatin-treated cells with 200 nmol/l bafilomycin A1 before acridine orange staining completely inhibited the acidification of vacuoles and did not display red fluorescence. Similarly, other inhibitors of autophagy LY294002 or 3MA also prevented formation of cisplatin-induced acidic autophagic vacuoles (data not shown).

Autophagy inhibition accelerates cisplatin-induced caspase activation and cell death. PI 3-kinase is an essential component of core machinery involved in autophagic vesicle formation
The inhibitors of PI 3-kinase wortmannin or LY294002 or 3MA block the formation of autophagy (5, 26, 32). Thus, we determined whether PI 3-kinase inhibitors promote cisplatin-induced caspase activation and apoptosis. Wortmannin (0.25 μM) resulted in an enhanced and early increase in cisplatin-induced caspase-3/7 and caspase-6 activity (cleavage of DEVD-AMC and VEID-AMC, respectively) compared with that of cisplatin alone in a time-dependent (0–24 h) manner (Fig. 6). Similar results were obtained with another autophagy inhibitor LY294002 (data not shown). Wortmannin (0.25 μM) alone (as shown in the figure) or LY294002 alone minimally affected caspase-3/7 (Fig. 6A) and caspase-6 activation (Fig. 6B), but this activation was insignificant compared with the activation by cisplatin alone or by wortmannin and cisplatin together. Thus, these studies indicate that inhibition of autophagy enhances cisplatin-induced caspase-3/7 and caspase-6 activation. In marked contrast, inhibition of autophagy did not affect the cisplatin-induced proinflammatory caspase-1 activity (data not shown). Since the activation of caspase-3 and -7 was determined together as they utilize the same DEVD-AMC substrate, we used an antibody specific to caspase-3 that identified only caspase-3 activation but not caspase-6 or caspase-7 activation by Western blot. As shown in Fig. 6C, proteolytic processing of pro-caspase-3 resulted in the formation of a 17-kDa subunit of the active caspase-3 which first appeared at 8 h of cisplatin exposure. The autophagic inhibitor LY294002 (10 μM) resulted in the appearance of active caspase-3 as early as 2 h of cisplatin treatment and enhanced activation at later time points (Fig. 6C). These data suggest that inhibition of the autophagy pathway results not only in earlier activation of executioner caspses but also accelerates this activation at later periods in cisplatin-induced injury to RTEC. The effect of inhibition of cisplatin-induced autophagy pathway was also reflected in early and enhanced apoptosis (Fig. 7). Thus, cisplatin-induced apoptosis is markedly enhanced by inhibition of autophagy.

Fig. 5. Detection of cisplatin (CP)-induced acidic autophagic vacuoles. LLC-PK1 cells were seeded in T-25 flasks at 70% confluence and were untreated or treated with 50 μM cisplatin in the presence or absence of 200 nM autophagic inhibitor bafilomycin A1 for various time points as indicated. At the appropriate time points as indicated, cells were incubated with 1 μg/ml acridine orange (Molecular Probes) in serum-free medium for 15 min. The acridine orange was removed and fluorescent micrographs were obtained using an inverted fluorescence microscope.
Downregulation of Beclin 1 and Atg5 by siRNA increases cisplatin-induced caspase activation and cell death. To investigate the specific functional role of key autophagy proteins Beclin 1 and Atg5 in cisplatin-induced autophagy, we used an RNAi approach for their specific inhibition. Inhibition of Beclin 1 and Atg5 using their respective siRNA (Fig. 8A) resulted in cisplatin-induced enhanced activation of caspase-3/7 and -6 (Fig. 8B). The effect of siRNA was specific, since scrambled siRNA did not decrease the expression of Beclin 1 and Atg5 (Fig. 8A). Beclin 1 and Atg5 inhibition also accelerated cisplatin-induced cell death as revealed by an MTT assay (Fig. 8C). We also confirmed that Beclin 1 and Atg5 siRNAs prevent autophagy. As shown in Fig. 8D, cisplatin-induced autophagic vacuoles, which displayed red fluorescence with acridine orange (red fluorescence at 6 and 16 h of cisplatin treatment), were inhibited by siRNA for Beclin 1 and Atg5.

Fig. 6. A: effect of an autophagic inhibitor on caspase-3/7 activation in cisplatin-induced injury to LLC-PK1 cells. Cells were treated with 50 μmol/l cisplatin in the presence (black bar) or absence (open bar) of wortmannin (0.25 μmol/l) for various time periods as indicated. Cells were also treated with wortmannin alone as shown. Caspase-3 activity in cell lysates (50 μg protein) was determined using the substrate DEVD-AMC. The incubations were as described in MATERIALS AND METHODS. Results are means ± SE (n = 4). *P < 0.005 compared with cisplatin-treated cells. B: effect of autophagy inhibitor on caspase-6 activation in cisplatin-induced injury to LLC-PK1 cells. Cells were treated with 50 μmol/l cisplatin in the presence (black bar) or absence (open bar) of wortmannin (0.25 μmol/l) for various time periods as indicated. Cells were also treated with wortmannin alone as shown. Caspase-6 activity in cell lysates (50 μg protein) was determined using the substrate VEID-AMC. Results are means ± SE (n = 4). *P < 0.025 compared with cisplatin-treated cells. C: effect of the autophagy inhibitor LY294002 on cisplatin-induced time course expression and proteolytic processing of caspase-3. Cells were treated with 50 μmol/l cisplatin in the presence of LY294002 (5 μmol/l) for various time periods as indicated. Cell lysates (100 μg protein) were subjected to Western blot using antibodies specific for caspase-3. As shown, an autophagic inhibitor induces early and accelerated activation of caspase-3.

Fig. 7. Effect of the autophagic inhibitor wortmannin on a cisplatin-induced time course of cell death. Cells were treated with 50 μmol/l cisplatin in the presence (black bar) or absence (gray bar) of wortmannin for the time points indicated. Following incubations, cell death was determined by the MTT assay as described in MATERIALS AND METHODS. Results are means ± SE (n = 5). *P < 0.001 compared with control cisplatin-treated cells.
Fig. 8. A: effect of siRNAs on cisplatin-induced Beclin 1 and Atg5. Cells were transfected with Beclin 1 and Atg5 siRNAs as described in MATERIALS AND METHODS and cell lysates were subjected to Western blots for Beclin 1 and Atg5. B: time course effect of Beclin 1 or Atg5 siRNA on caspase-3/7 and -6 activation in response to cisplatin injury. LLC-PK1 cells were transfected with Atg5 or Beclin 1 siRNA or control-scrambled siRNA and treated with and without cisplatin (50 μM) as indicated. Cell lysates were prepared and analyzed for caspase-3/7 and caspase-6 activation as described in MATERIALS AND METHODS. *P < 0.02 compared with CP-treated cells. C: time course effect of Beclin 1 or Atg5 siRNA on cisplatin-induced cell death. LLC-PK1 cells were transfected with Atg5 or Beclin 1 siRNA or control-scrambled siRNA and treated with and without cisplatin (50 μM) as indicated. Following incubations, cell death was determined by the MTT assay as described in MATERIALS AND METHODS. *P < 0.05 compared with untreated control. D: effect of siRNA for Beclin 1 and Atg5 on formation of autophagic vacuoles as revealed by acridine orange. LLC-PK1 cells were transfected with Atg5 or Beclin 1 siRNA or control-scrambled siRNA and treated with and without cisplatin (50 μM) as indicated. Cells were incubated with 1 μg/ml acridine orange in serum-free medium for 15 min. The acridine orange was removed and fluorescent micrographs were obtained using a fluorescence microscope.
DISCUSSION

The original discovery of autophagic genes (Atg) in yeast has led to the discovery of several mammalian orthologs and subsequently has increased our knowledge of mammalian autophagy and its role in cell survival or death decision. This suggests that the autophagy pathway is highly conserved from yeast to human (18, 19). Among the various mechanisms that regulate cell death (including apoptosis and necrosis), autophagy has emerged as another major programmed mechanism to control life and death, much like programmed cell death is for apoptosis in multicellular organisms.

The present study provides evidence for the first time for the occurrence of autophagy and its role in RTEC in response to cisplatin. The autophagy response to cisplatin was identified by specific characteristic hallmarks of autophagy including induction of LC3-I, accumulation of lipid-conjugated LC3-II protein, formation of punctated staining of autophagosome-associated LC3-II, acidic autophagic vacuoles, autophagolysosomes, and upregulation of critical autophagic proteins Atg5 and Beclin 1. We demonstrated that autophagy is an immediate response to cisplatin injury and is required for cellular adaptation against cisplatin-induced stress conditions. These studies demonstrate that autophagy promotes cellular survival during a cisplatin-induced preapoptotic lag phase, resulting in a delayed apoptotic response. Induction of autophagy during the initial period of cisplatin insult may provide an appropriate environment to maintain cellular homeostasis before reaching the threshold for cisplatin-induced apoptosis. Thus, at the initial stages of cisplatin injury, autophagy may function efficiently in eliminating unwanted or damaged organelles and other cytoplasmic macromolecules to establish cellular homeostasis.

We identified that critical autophagic proteins that participate in the development of early stages of autophagy (Fig. 9) are upregulated in response to cisplatin injury. One of the early characteristic features is the conversion of a soluble LC3-I form to its lipid-conjugated form, LC3-II, which involves a ubiquitin-like conjugation system (30, 35). LC3-I is a mammalian ortholog of yeast Atg8 protein and, on induction and conversion to LC3-II, it is utilized as an essential component in the vesicle elongation process during the formation of an autophagosome, which has a double membrane structure (Fig. 9). The fluorescence microscopy and Western blot analysis indicated that LC3-II conversion is initiated very early during cisplatin treatment and remains associated with apoptosis.

In most autophagy studies, LC3-II formation is the most commonly used marker of autophagy. Its conjugation with PE lipid and subsequent specific recruitment to the membrane vesicle autophagosome enables its identity by an electrophoretic mobility shift in SDS gels and by a fluorescent staining pattern that changes from diffuse cytosolic to punctate form when GFP-LC3 is used in the studies. Beclin 1 protein is a mammalian ortholog of Atg6 protein. Both Beclin1/Atg6 and Atg5 proteins are upregulated in response to cisplatin. Beclin 1 was originally identified as a Bcl-2-interacting protein, but later studies showed that the major physiological partner for Beclin 1 is the mammalian class III PI 3-kinase Vps34. Beclin1/Atg6 is involved in the formation of a PI 3-kinase multiprotein complex during the vesicle nucleation in the initial stage of autophagosome formation (7, 36). In the multiprotein complex, Beclin 1/Atg6 is involved in the activation of Vps34, a class III PI 3-kinase, to generate PI-3 phosphate. Atg5 along with Atg12 were the first mammalian autophagy genes identified that participate in the autophagosome formation (28). During vesicle elongation stage of autophagosome formation (Fig. 9), Atg5 and Atg12 are covalently conjugated by a ubiquitin-like conjugation system with the help of the E1-like enzyme Atg7 and the E2-like enzyme Atg10 (36). A recent study suggested that truncated Atg5 formed by cleavage of Atg5 by calpain 1 and 2 is translocated to the mitochondria and causes cytochrome c release and cell death (39). Some low level of Beclin 1 and Atg5 expression suggests a basal level of autophagy under normal conditions that may trigger a rapid autophagic response under stress conditions.

We provide evidence that autophagy is an early response to cisplatin injury, is induced at the preapoptotic lag phase, and persists during the injury. Inhibition of autophagy by specific inhibitors switches autophagy to apoptotic cell death, suggesting that autophagy plays a defense against cisplatin. We demonstrated that inhibition of autophagy by wortmannin or LY294002, inhibitors of PI 3-kinase, blocks cisplatin-induced autophagy and enhances activation of executioner caspases-3, -6, and -7. These inhibitors had no effect on the activation of proinflammatory caspase-1. The inhibition of autophagy explains the delayed response of cisplatin to caspase activation.

Fig. 9. Autophagy pathway depicting initiation, execution, and maturation of autophagy. The distinct stages of the autophagy pathway are controlled by Atg gene products. The vesicle nucleation and vesicle elongation process in the initial stages of autophagy sequesters the cytosolic contents and results in the formation of the autophagosome, a double-membraned vesicle. The outer membrane of the autophagosome subsequently fuses with the lysosome to form an autolysosome and the internal contents of the autophagosome are then degraded by lysosomal hydrolases.
and cell death. Cisplatin-induced upregulation of Beclin 1 and Atg5 demonstrates that their expression is necessary for the suppression of apoptosis. Our studies show that downregulation of Beclin 1 or Atg5 abrogates the autophagic prosurvival response and allows cells to die by apoptosis. Other DNA-damaging agents camptothecin and etoposide, which, like cisplatin, activate an intrinsic pathway of cell death, were recently shown to induce autophagy that delays apoptosis and plays a prosurvival role in MCF-7 cells (1). A protective role of autophagy has also been reported under oxidative stress. Inhibition of autophagy causes increased cell death in cardiac myocytes after anoxia-reoxygenation injury (10). Rapamycin, a lipophilic, macrolide antibiotic, induces autophagy by inactivating the protein mammalian target of rapamycin (mTOR). Rapamycin was shown to protect cells in response to various apoptotic stimuli (33).

In summary, we provided evidence that autophagy is induced early at the preapoptotic lag phase during cisplatin injury to RTEC. Cisplatin-induced autophagy suppresses apoptosis as inhibition of autophagy accelerates apoptotic cell death. We find that autophagy induction is a defense response against cellular stress induced by cisplatin. Our studies suggest that the autophagy pathway may play an important role in cell survival in response to acute kidney injury.

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REFERENCES


