Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging chemotherapy

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Running head: autophagy and DNA-damaging chemotherapy in MM

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Translational Relevance

Multiple myeloma (MM) is a hematologic malignancy resulting from a clonal proliferation of plasma cells in the bone marrow. Although there have been major advances in the treatment of MM in recent years, it remains incurable mostly because of the development of drug resistance. Herein, we demonstrate that DNA-damaging agents (such as doxorubicin and melphalan) induced autophagy as a prosurvival mechanism in MM cells by engaging Bcl-2/Beclin1/Class III phosphatidylinositol 3-kinase complex. Importantly, targeting suppression of autophagy by minimally toxic concentration of pharmacological inhibitor hydroxychloroquine (which has been safely used worldwide for treatment of malaria and autoimmune disease) dramatically augmented proapoptotic activity of DNA-damaging chemotherapy both in vitro using MM cell lines or purified patient MM cells and in vivo in a human plasmacytoma xenograft mouse model. These data thus provide a rationale for clinical evaluation of hydroxychloroquine in combination with DNA-damaging chemotherapy in MM.
Abstract

Purpose: Although autophagy occurs in most tumor cells following DNA damage, it is still a mystery how this DNA-damaging event turns on the autophagy machinery in multiple myeloma (MM), and how the functional status of autophagy impacts on its susceptibility to death in response to DNA-damaging chemotherapy.

Experimental Design: We investigate the effects of DNA damage on autophagy in MM cells, and elucidate its underlying molecular mechanism. Then, we examined the impacts of pharmacological or genetic inhibition of autophagy on DNA damage-induced apoptosis. Further, the antmyeloma activity of autophagy inhibitor in combination with DNA-damaging agents was evaluated in MM xenograft models.

Results: we demonstrated that DNA-damaging drugs, doxorubicin and melphalan, induce caspase-dependent apoptosis and concurrently trigger Beclin 1-regulated autophagy in human MM cell lines H929 and RPMI 8226. Mechanistically, association of autophagy–execution proteins Beclin 1 with Class III phosphatidylinositol 3-kinase, which is inhibited by Bcl-2 recruitment, contributes directly to the autophagic process. Importantly, targeting suppression of autophagy by minimally toxic concentrations of pharmacological inhibitors (hydroxychloroquine and 3-methyladenine) or short hairpin RNAs against autophagy genes Beclin 1 and Atg5 dramatically augments proapoptotic activity of DNA-damaging chemotherapy both in vitro using MM cell lines or purified patient MM cells and in vivo in a human plasmacytoma xenograft mouse model.

Conclusion: These data can help unravel the underlying molecular mechanism of autophagy in DNA-damaged MM cells, and also provide a rationale for clinical evaluation of autophagy inhibitors in combination with DNA-damaging chemotherapy in MM.
Introduction

Engulfment of cytoplasmic material into specialized double-membrane vesicles known as autophagosomes is the defining feature of a process referred to as macroautophagy or simply autophagy (1-3). Subsequent fusion of autophagosomes with the endolysosomal network leads to hydrolytic degradation of the sequestered material (4). Autophagy occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover (4, 5). It is induced to high levels when cells are preparing to rid themselves of damaging cytoplasmic components during metabolic and therapeutic stress (4, 5). Importantly, a number of anticancer therapies, including DNA-damaging chemotherapy have also been observed to induce the accumulation of autophagosomes in tumor cell lines in vitro (6-10). Further, genetic knockdown of phylogenetically conserved autophagy-related (Atg) genes, such as Beclin 1 and Atg5, usually accelerates, rather than prevents, cell death in these settings (8). Thus, it seems plausible that autophagy induction seen in response to antineoplastic therapies mostly represents a prosurvival mechanism activated to counteract the deleterious effects of endogenous metabolic stress and possibly also treatment on tumor cells (11-18).

Multiple myeloma is a hematologic malignancy resulting from a clonal proliferation of plasma cells in the bone marrow. Despite clinical efficacy of high-dose therapy as well as novel drugs including thalidomide (19, 20), lenalidomide (19, 20), and bortezomib (21-24) in patients with MM, responses are not durable and few, if any, patients are cured, which highlights the dire need for novel therapeutic strategies to improve patient outcome. Curiously, an earlier study suggested a role for autophagy as a potential prosurvival mechanism in MM cells (11), which is reminiscent of the cytoprotective function for autophagy in cancer chemotherapy (12-14). Thus, it seems that targeting autophagy pathway can be exploited in treatment of MM. However, the role of autophagy in MM appears more complex than hitherto believed and much work is still required to decipher the underlying molecular underpinnings of autophagy in MM, and define how the functional status of autophagy in MM impacts on its response to treatment.
In the present study, we characterized the effects of autophagy inhibition on anticancer potency of DNA-damaging drugs doxorubicin and melphalan, which are front-line chemotherapeutic drugs for the treatment of MM. Here we showed that both drugs induced Beclin 1-regulated autophagy in H929 and RPMI 8226 cells, respectively. Furthermore, association of Beclin 1 with Class III phosphatidylinositol 3-kinase (PI3K), which was inhibited by antiapoptotic protein Bcl-2 recruitment, contributed directly to the autophagic process. Importantly, suppression of autophagy via either pharmacological inhibitors or short hairpin RNAs (shRNAs) against Beclin 1 or Atg5 significantly augmented in vitro and in vivo antamyeloma activity of DNA-damaging chemotherapy. Collectively, these data provide compelling evidence that interactions between Beclin 1, Class III PI3K and Bcl-2 involve the modulation of autophagy in DNA-damaged MM cells, and also provide a rationale for clinical evaluation of autophagy inhibitors in combination with DNA-damaging chemotherapy in MM.

Materials and Methods

Reagents

The following reagents were used: anti-cleaved-caspase-3, anti-KI-67 antibodies, rapamycin (Cell Signaling Technology); anti-Class III PI3K, anti-Bcl-2, anti-Beclin 1, anti-Atg5, anti-LC3 antibodies (Abcam); anti-β-actin antibody, hydroxychloroquine (HCQ), 3-methyladenine (3-MA), bafilomycin A1 (BafA1), melphalan, doxorubicin (Sigma-Aldrich).

Cells and cell culture

Human MM cell lines H929 and RPMI 8226 (American Type Culture Collection) were maintained as previously described (25). Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy individuals, and patient MM cells were isolated from bone marrow samples using CD138 MicroBeads and the MidMACS magnetic cell separator according to the manufacturer's protocol (Miltenyi Biotec). Informed consent was obtained from all patients in accordance with an approved Xijing Hospital Institutional Review Board (IRB) protocol and in accordance with the Declaration of Helsinki.
Cell viability and apoptosis assays

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assays. Annexin V/propidium iodide (PI) staining assays were performed as previously described (25). A TUNEL apoptosis detection kit (Roche Diagnostics) was used to measure apoptosis in murine tumor sections per manufacture’s instructions.

Transmission electron microscopy

Cells were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 1 hour at 4°C. The samples were washed, then fixed with 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 1 hour at 4°C. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and then examined with a JEM2000EX transmission electron microscope (JEOL) at an accelerating voltage of 80 kV.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer as described (25), the cleared lysates (400-500 μg total protein) incubated for 2 hours with 2-3 μg of anti-Beclin 1 antibody or control IgG, and the immune complexes captured over 50 μl of Protein A Sepharose 50% slurry (Amersham Biosciences). Antigen-antibody complexes were released from the beads with a 10-minute incubation at 95°C. Proteins were subjected to 12% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto PVDF membranes (Millipore). After blocking, filters were incubated sequentially with appropriate primary and horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). The bound antibodies were visualized by chemiluminescence using SuperSignal reagent (Pierce).

Lentivirus-mediated gene knockdown

The pLentilox 3.7 (pLL3.7) vector (a gift from Jinyi Zhang, Samuel Lunenfeld institute, University of Toronto), carries a cytomegalovirus promoter driving expression of enhanced green fluorescent protein (eGFP) and the mouse U6 promoter for downstream shRNA synthesis (26). The sequences, 5’-GCACTCTGGATGGGATTG-3’ (27) or
5'-CAGTTTGGCACAATCAATA-3' (28), known to silence human Atg5 or Beclin 1 as a small interfering RNA (siRNA), was adapted with a loop sequence (29) and then inserted into pLL3.7 vector to create a shRNA for expression of siRNA. Lentiviruses were generated as described by us previously (25), and the titers ranging from 4×10⁶ to 10×10⁶ viral units/mL were usually obtained. To high efficiently infect MM cells, 1×10⁶ cells were cultured in 1mL of Reduced Serum Medium (Invitrogen) supplemented with 10-50×10⁶ lentiviral particles (multiplicity of infection, 10-50) and 10 µg/mL Polybrene (Sigma-Aldrich). Three days after infection, shRNA-expressing cells were collected by sorting of eGFP-positive cells.

**Human plasmacytoma xenograft model**

Approval of these studies was provided by the Institutional Animal Care and Use Committee at the Fourth Military Medical University. The xenograft tumor model was performed as previously described (12, 30, 31). CB-17 severe combined immunodeficiency (SCID) mice (SLAC, Laboratory Animal, Shanghai, China) were subcutaneously inoculated in the flanks with H929 cells (3 x 10⁷/mouse) or RPMI 8226 cells (1 x 10⁷/mouse) in 100 µL serum-free RPMI-1640 medium. When tumors were measurable approximately 2 weeks after MM cell injection, mice were divided into 6 groups (n=3 per group): control (PBS) group, groups treated with HCQ or doxorubicin or melphalan alone, and groups treated with HCQ/doxorubicin or HCQ/melphalan combinations. HCQ was injected i.p. at a dose of 60 mg/kg daily for 14 days; melphalan was given i.p. at 3.5 mg/kg daily for 7 days; doxorubicin was injected i.p. at 3 mg/kg on the first day of treatment, and then administered once more at 4-day interval. Tumor size was measured every alternate day using a caliper, and tumor volume was calculated using the formula: \( V = 0.5a \times b^2 \), where "a" and "b" are the long and short diameter of the tumor, respectively. All animals were sacrificed individually by CO₂ asphyxiation. In situ detection of apoptosis in xenografted MM tumors was carried out by using TUNEL assays and hematoxylin and eosin (H&E) staining. Ki-67 expression was determined by automated immunohistochemical staining to quantify proliferation.

**Statistical analysis**
All experiments, except immunoblots, were performed in triplicate. Student's t test was used to analyze the apoptosis data. For all studies, the level of statistical significance was set at $P < 0.05$.

Results

Autophagy is activated concurrently with apoptosis in DNA-damaged MM cells

For these studies, H929 or RPMI 8226 cells were exposed for 24 hours to various concentrations of doxorubicin and melphalan, respectively, and then analyzed by MTT assay for $IC_{50}$ values (Supplementary Fig. S1A and B). Importantly, drug-induced decrease in viability is due mainly to apoptosis, as determined by annexin V/PI staining assays (Supplementary Fig. S1C). To date, a number of antineoplastic therapies, including DNA-damaging drugs (such as camptothecin and etoposide), have been shown to activate autophagy at low doses (6-8). To assess the effects of either doxorubicin or melphalan on autophagic process in MM, subsequent in vitro experiments were carried out by using both drugs at concentrations much lower than their respective $IC_{50}$ for each MM cell line, thereby enabling a model in which cells were mainly in the stage of activation of autophagy rather than induction of apoptosis. For this purpose, both H929 and RPMI 8226 cells were exposed for 24 hours to lower doses of doxorubicin (0.5 and 0.25 $\mu$mol/L, respectively) or melphalan (10 and 5 $\mu$mol/L, respectively), which only resulted in 5-10% apoptotic cell death (Supplementary Fig. S1C), and then analyzed for autophagy. It has been demonstrated that Atg protein LC3 during autophagy is processed to a cytosolic version (LC3-I, 18 KDa) and then converted to a lipidized form (LC3-II, 16 KDa) that stably associates with the membrane of phagophores (a preautophagosome structure) and autophagic vacuoles (e.g., autophagosomes or autophagolysosomes) (32). Therefore, autophagy can be detected morphologically (by observing formation of autophagic vacuoles) and biochemically (by assessing generation of LC3-II) (33, 34). Consequently, electron microscopic studies revealed that most of doxorubicin- or melphalan-treated MM cells displayed viable attributes and contained numerous autophagic vacuoles with undegraded or partially degraded contents.
(Fig. 1Aa-b and B), whereas untreated cells exhibited few such features (Fig. 1Ac and B). As a control, many autophagic vesicles were also noted following exposure of cells to the known autophagy inducer rapamycin (Fig. 1Ad and B). As is consistent with these findings, treatment with either drug also caused a time-dependent conversion of LC3-I to LC3-II (Fig. 1C and D). Thus, these data together suggest the activation of autophagic response in doxorubicin- or melphalan-treated MM cells. Nevertheless, there is a need to further discriminate between two physiologically distinct scenarios—increased autophagic flux without impairment in autophagic turnover versus impaired clearance of autophagosomes (15). For these studies, we next exposed MM cells to either drug in the presence of 10 mmol/L 3-MA (a Class III PI3K inhibitor widely used to disrupt formation of autophagosomes) or 8 μmol/L HCQ (a lysosomotropic weak base). Surprisingly, however, 3-MA exposure markedly inhibited drug-induced autophagic vacuole formation (Fig. 1Ae-g and B) but failed to result in a parallel decrease in LC3-II amounts (Fig. 2A and B). Our results are in line with the recent reports showing that 3-MA blocks autophagy but does not completely inhibit LC3-II conversion in chemical agent-treated human neuroblastoma cells (35), and moreover a normal levels of LC3-II is not sufficient evidence for autophagy (34). Thus, it appears that 3-MA compromises DNA damage-induced autophagy not only via inhibiting LC3-II formation (33, 34), but also through disrupting phagophore development into autophagic vacuoles (herein, LC3-II amounts largely reflect levels of phagophore), given that LC3-II is located to phagophores as well as completed autophagosome (32). By contrast, HCQ cotreatment significantly increased amounts of autophagic vacuoles (Fig. 1Ah-j and B) and facilitated LC3-II accumulation (Fig. 2C and D). Similar results were also obtained with 10 nmol/L BafA1 (an inhibitor of vacuolar-type ATPase) (Supplementary Fig. S2A and B). Given that both HCQ and BafA1 can increase the pH of acidic vesicles, thereby preventing the fusion of autophagosomes with lysosomes (36), our data suggest that doxorubicin and melphalan induce autophagy-associated changes in MM cells primarily due to enhanced induction of autophagy rather than compromised clearance of autophagosomes.

**Functional interactions between Beclin 1, Class III PI3K and Bcl-2 involve the modulation of autophagy in DNA-damaged MM cells**
To further investigate the molecular mechanisms through which doxorubicin and melphalan promote autophagy, we next focused on the early steps of autophagosome nucleation in which Beclin 1 participates (2, 4). Curiously, our results showed that treatment with either single drug resulted in a time-dependent increase in levels of Beclin 1 protein (Fig. 3A), providing further support for the critical role of Beclin 1 in mediating autophagy in DNA-damaged MM cells. It has been demonstrated that Beclin 1 forms a complex with Class III PI3K, and stimulates formation of autophagosomes by promoting activation of Class III PI3K (37, 38). Furthermore, the capacity of Beclin 1 to induce autophagy can be suppressed by Bcl-2 through direct binding (39-42). Thus, it is tempting to speculate that this PI3K complex may involve regulation of autophagy in DNA-damaged MM cells. To address this possibility, Beclin 1 immunoprecipitates from doxorubicin- or melphalan-treated cells were evaluated for the presence of either Class III PI3K or Bcl-2. As seen in Fig. 3B and C, amounts of Class III PI3K associated with Beclin 1 were substantially increased, whereas Bcl-2 binding to Beclin 1 was markedly reduced. Furthermore, effects of either drug on Class III PI3K or Bcl-2 binding to Beclin 1 were disrupted by 3-MA (Fig. 3B). Consistent with the reported dependency of PI3K activity on Beclin 1 expression (43), these observations thus suggest that Beclin 1-Class III PI3K interaction, which is inhibited by Bcl-2 recruitment, may contribute directly to doxorubicin- or melphalan-induced autophagic response in MM cells. Interestingly and mysteriously, lysosomal inhibitor HCQ promoted Class III PI3K binding to Beclin 1 and concomitantly suppressed Bcl-2 recruitment in DNA-damaged MM cells (Fig. 3C), which are largely presumed to represent a compensatory response to impaired clearance of autophagosomes, although direct data proving this concept are lacking.

**Pharmacological inhibition of autophagy enhances induction of apoptosis in DNA-damaged MM cells**

As indicated above, doxorubicin and melphalan activated an autophagic response, while simultaneously triggering apoptosis in MM cells. Considering that autophagy may function as a prosurvival mechanism in MM cells (11), we were next interested in testing the hypothesis that disrupting autophagy pathway would enhance proapoptotic effects of DNA damaging
drugs. To this end, the cells were treated for 24 hours with low concentrations of either drug in the presence or absence of 3-MA (10 mmol/L) or HCQ (8 μmol/L). A dose-dependent induction of apoptosis, as determined by annexin V/PI staining assays, was observed and this was especially profound in cells treated with HCQ/doxorubicin, HCQ/melphalan, 3-MA/doxorubicin or 3-MA/melphalan combinations, compared with cells treated with either single drug (Fig. 4A). These observations strongly suggest that minimally toxic concentrations of autophagy inhibitors markedly potentiate the lethality of DNA-damaging drugs in MM cells. To further characterize the apoptotic program triggered by these combination regimens, we next examined the proteolytic processing of procaspase-3. As revealed by immunoblotting analysis (Supplementary Fig. S3A-D), this hallmark of apoptosis was most pronounced when either drug was combined with 3-MA or HCQ, consistent with the changes in percentage of apoptotic cells (Fig. 4A). Moreover, caspase-3 activation induced by the combination treatments were markedly attenuated by means of broad spectrum caspase inhibitor z-VAD-fmk (Supplementary Fig. S3A-D), suggesting that both drugs induce caspase-dependent apoptosis in MM cells much more effectively when autophagy pathway is disrupted. To further evaluate the efficacy of autophagy inhibitors in combination with either doxorubicin or melphalan in a clinically relevant scenario, we investigated their effects against primary MM cells obtained from 21 newly diagnosed patients. Similarly, the anticancer activity of either drug against these primary MM cells was significantly enhanced in the presence of 3-MA or HCQ (Fig. 4B). Surprisingly, moreover, in primary MM cells from 11 heavily pretreated patients who failed to respond to several treatments including doxorubicin and melphalan, autophagy inhibitors still retained their ability to enhance proapoptotic effects of both drugs (Fig. 4B), indicating the potential utility of these combination regimens in MM patients who had shown clinical resistance to conventional therapies. In contrast, PBMCs from healthy donors treated under the same conditions displayed significantly less induction of apoptosis than patient MM cells, providing further support for the selectivity of these treatments (Fig. 4C). Collectively, these data therefore rather suggest that both doxorubicin and melphalan stimulate autophagy as a cytoprotective mechanism and that inhibiting this process may be an effective strategy to augment DNA damaging drug-induced apoptosis in MM cells.
Knockdown of Beclin 1 or Atg5 sensitizes MM cells to DNA damaging drug-induced apoptosis

Chemical suppression of autophagy might be affected by nonwarranted side effects of drugs endowed with limited selectivity. To specifically inhibit autophagic process and further validate our observations, we next employed a lentivirus-mediated RNA interference (RNAi) approach to silence the essential Atg genes such as Beclin 1 and Atg5 (2, 4, 44), which were also induced by either doxorubicin or melphalan in a time-dependent manner (Figs. 3A and 5A). We generated MM cells functionally deficient in Beclin 1 or Atg5 by infecting cells with lentivirus producing a shRNA specific for Beclin 1 or Atg5 gene. Knockdown of Beclin 1 or Atg5 gene expression in shRNA-expressing cells, which were collected by sorting of eGFP-positive cells, was confirmed by immunoblotting (Fig. 5B and C). The protein levels of either Beclin 1 or Atg5 were reduced by an average of 90%, compared with those in vector control cells (Fig. 5B and C), consistent with a previous report showing that one or more RNAi expression construct should give 40%-90% reduction in gene expression when used transiently (45). More importantly, disabling Beclin 1 or Atg5 expression disrupted constitutive or DNA damaging drug-induced conversion of LC3-I to LC3-II (Fig. 5B and C), in agreement with the studies showing that Beclin 1-PI3K complex affects LC3 processing (43), and that Atg12-Atg5 conjugates has E3 ligase-like activity for LC3 protein lipidation (46). These findings thus reveal that Beclin 1 or Atg5 deficiency results in a specific inhibition of autophagy in MM cells. To further explore effects of inactivation of either Beclin 1 or Atg5 gene on DNA damaging drug-induced apoptosis, the shRNA-expressing cells were treated with doxorubicin and melphalan, respectively. Not surprisingly, an enhanced apoptosis was observed in the cells with either Beclin 1 or Atg5 deficiency when compared with vector control cells (Fig. 5D), while no significant effects on cell viability were observed at baseline (Fig. 5D). These data therefore suggest that genetic inhibition of autophagy can precipitate doxorubicin- or melphalan-triggered apoptotic cell death and further confirm cytoprotective potential of autophagy in DNA-damaged MM cells.
Autophagy inhibition potentiates in vivo antitymoma activity of DNA-damaging chemotherapy

HCQ has been used worldwide for treatment of malaria and autoimmune disease for more than 60 years, whereas 3-MA is unsuitable for in vivo use. To further investigate the therapeutic efficacy of manipulating autophagy, we therefore focused our subsequent studies on either HCQ/doxorubicin or HCQ/melphalan combinations due to their potential clinical relevance. Having shown that HCQ enhances doxorubicin or melphalan-induced apoptosis in MM cells in intro, we next examined in vivo efficacy of HCQ using a human plasmacytoma xenograft mouse model (12, 30, 31). When the H929 tumors were measurable, mice were matched for tumor volumes and randomly assigned to receive HCQ, doxorubicin or melphalan, or a combination of HCQ with either drug. As seen in Fig. 6A and B, HCQ treatment had minimal effect on the growth of tumors, which increased as in control mice, whereas treatment with either doxorubicin or melphalan displayed rather moderate antitumor activity. However, when HCQ was combined with either drug, there was a significant (70% to 75%) reduction in tumor growth relative to untreated mice (Fig. 6A and B, insets), and the extent of tumor growth inhibition was similar in mice treated with HCQ/doxorubicin (Fig. 6A) versus mice treated with HCQ/melphalan (Fig. 6B). Furthermore, no significant weight loss was observed even after 3 to 4 weeks of combination treatments (data not shown). Similar results were obtained using RPMI 8226 tumors (supplementary Fig. S4A and B). These findings taken together suggest that HCQ potently enhances in vivo antitymoma activity of either doxorubicin or melphalan, and that the combination regimens markedly reduce tumor growth and are well tolerated in vivo.

We next investigate the effect of the drug combinations on in vivo apoptosis using TUNEL and H&E staining of paraffin-embedded sections of xenografted tumors. The peripheral necrotic regions of the sections from the xenograft were excluded for quantification of cell death. As shown in Fig. 6C, either single drug caused a modest increase in the number of TUNEL-positive cells (brown color) compared with control cohorts. However, the HCQ/doxorubicin and HCQ/melphalan combinations dramatically increased the number of...
TUNEL-positive cells compared with either treatment alone (Fig. 6C). Paralleling these data, the morphological changes characteristic of apoptosis (such as chromatin condensation) along with a marked decrease in Ki-67+ cells was also noted in tumor sections from mice treated with HCQ/doxorubicin (Fig. 6D) or HCQ/melphalan (data not shown), relative to tumors from mice receiving vehicle. Together, these data further identify the apoptosis induction as a major component of the mechanism underlying HCQ’s ability to potentiate antmyeloma effects of DNA-damaging chemotherapy.

Discussion

As any other major phenomenon of cell biology (such as division, differentiation and cell death), autophagy can also be perturbed in cancer cells and modulated by anticancer chemotherapies (7, 14, 15). However, very little is known about molecular details of autophagy regulation in MM cells, and how the functional status of autophagy in MM impacts on its response to DNA-damaging chemotherapy. This study, for the first time, shows that low doses of doxorubicin or melphalan induced a Beclin 1-regulated autophagic response in MM cells. Recently, it was reported that Beclin 1 facilitates formation of autophagosomes via its recruitment to Class III PI3K multiprotein kinase complex (37, 38). The autophagy-promoting activity of Beclin 1 is also suppressed by antiapoptotic members of Bcl-2 family through direct binding (39, 42), because that Beclin 1 is a bona fide BH3-domain-only protein, and that amphipathic α-helix of its BH3 domain binds to the conserved hydrophobic groove of Bcl-2 similarly to the interactions shown previously for other BH3-domain-only proteins (39-42).

Under normal steady-state growth conditions, Beclin 1 is bound to various Bcl-2 family members, whereas its dissociation from Bcl-2 mediates autophagy (39). Based on these premises and incognita, we therefore next explore the role for Class III PI3K complex in governing autophagy in DNA-damaged MM cells. Our results showed that during autophagy in response to low doses of doxorubicin or melphalan, Bcl-2 binding to Beclin 1 was significantly reduced, whereas Class III PI3K binding to Beclin 1 was markedly enhanced. Given that Beclin 1-regulated autophagy can be inhibited by overexpression of Bcl-2 (39), which also causes a disruption of Beclin 1-Class III PI3K interaction (39), these data suggest a model in which Beclin 1-regulated autophagy triggered by doxorubicin and melphalan,
respectively, is negatively regulated by Bcl-2, and positively regulated by Class III PI3K. These data also suggest that a decrease in Bcl-2 binding to Beclin 1 in response to either drug may lead to an increase in amounts of Class III PI3K associated with Beclin 1, thereby promoting autophagic process in DNA-damaged MM cells.

Another issue that has been raised and discussed in the literature is that in many cellular settings, autophagy accompanies, rather than causes, cell death. This argument is based on the fact that many studies that claim autophagic cell death prove that autophagy occurs, and that death ensues, but do not rigorously show that one causes the other (8-10). In other words, enhanced autophagy seems to constitute a failed attempt to adapt to stress and to survive, rather than a lethal catabolic process (14-17). These scenarios are again reminiscent of the potential prosurvival function of autophagy in MM (11), and raise the possibility that manipulating autophagy might modulate susceptibility of MM cells to apoptotic stimuli. To address this issue, current research efforts were focused on determining whether autophagy compromise unmasks or accelerates the apoptotic components of the DNA-damaging response in MM cells. Because low doses of doxorubicin or melphalan induced both autophagy and apoptosis simultaneously, we focused our subsequent analyses regarding whether disrupting autophagy program that was activated with apoptosis affects cell death in DNA-damaged MM cells. Our results revealed that pharmacological or genetic inhibition of autophagy markedly augmented drug-triggered apoptotic cell death in MM cells in vitro, which was further evidenced by using a human plasmacytoma xenograft mouse model. These data are concurrent with previous studies showing that autophagy delays the onset of apoptosis in breast cancer cells following DNA damage (47), and that autophagy inhibition with chloroquine enhances alkylating drug-induced cell death and lymphoma regression in mice (12). These data also support the assertion that autophagy may function as an adaptive response that allows MM cells to survive a DNA-damaging stimulus that would otherwise lead to their demise.

As mentioned above, autophagy inhibitors may be important adjuncts to enhance antmyeloma efficacy of existing DNA-damaging chemotherapy without potentiating toxicity.
Currently, few inhibitors of autophagy are well characterized, and most do not have favorable pharmacologic properties. The exception is the antimalarial HCQ, which has been more safely used than chloroquine for decades in patients for malaria prophylaxis and for treatment of rheumatoid arthritis (48). Importantly, the dose of HCQ used in present study is achievable clinically because HCQ at maximally tolerated doses used in rheumatoid arthritis may achieve a peak blood concentration approaching 10 μmol/L(49). Likewise, the results presented herein confirmed that systemic administration of HCQ at doses roughly equivalent to human doses used to treat malaria or rheumatoid arthritis was well tolerated for up to 20 days in mice; HCQ cotreatment with doxorubicin or melphalan did not result in additional toxicity in treated animals. Collectively, these data provide a platform for future studies to explore these combinations for the treatment of MM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Legends

**Figure 1.** Effects of doxorubicin or melphalan on autophagosome accumulation and LC3 processing. **A** and **B,** H929 cells were treated for 24 hours with (a) doxorubicin (Dox; 0.5 μmol/L), (b) melphalan (Mel; 10 μmol/L), (c) PBS (control), (d) rapamycin (Rap; 0.25 μmol/L), (e) 3-methyladenine (3-MA; 10 mmol/L), (f) 3-MA/Dox, (g) 3-MA/Mel, (h) hydroxychloroquine (HCQ; 8 μmol/L), (i) HCQ/Dox, or (j) HCQ/Mel. **A,** Electron microscopy pictures were taken. Autophagic vesicles are denoted by arrows. Scale bars: 1 μm. Original magnification, ×10,000. **B,** Quantification of autophagic vesicles per nonapoptotic cell was determined. Cells were considered viable or nonapoptotic if the integrity of the nuclear and cytoplasmic membrane was maintained. The data obtained from a minimum of 50 independent cells were averaged (mean ± SD.). *, P < 0.05. **C** and **D,** Effects of doxorubicin or melphalan on conversion of LC3-I to LC3-II. H929 and RPMI 8226 cells were treated for given times with (C) Dox (0.5 and 0.25 μmol/L, respectively) or (D) Mel (10 and 5 μmol/L, respectively) in the presence or absence of 3-MA (10 mmol/L) or HCQ (8 μmol/L). Protein lysates were subjected to immunoblotting with the indicated antibodies.

**Figure 2.** Effects of autophagy inhibitors on doxorubicin- or melphalan-induced LC3 processing. H929 and RPMI 8226 cells were treated for 24 hours with doxorubicin (Dox; 0.5 and 0.25 μmol/L, respectively) or melphalan (Mel; 10 and 5 μmol/L, respectively) in the presence or absence of (A and B) 3-methyladenine (3-MA; 10 mmol/L) or (C and D) hydroxychloroquine (HCQ; 8 μmol/L). Treatment with rapamycin (Rap; 0.25 μmol/L) served as positive controls. Protein lysates were subjected to immunoblotting with the indicated antibodies.

**Figure 3.** Effects of doxorubicin or melphalan on Beclin 1 expression and Beclin 1 association with Class III PI3K or Bcl-2 proteins. **A,** H929 and RPMI 8226 cells were treated for given times with (left) doxorubicin (Dox; 0.5 and 0.25 μmol/L, respectively) or (right) melphalan (Mel; 10 and 5 μmol/L, respectively). Protein lysates were subjected to immunoblotting with the
indicated antibodies. B and C, H929 and RPMI 8226 cells were treated for 24 hours with Dox (0.5 and 0.25 μmol/L, respectively) or Mel (10 and 5 μmol/L, respectively) in the presence or absence of (B) 3-methyladenine (3-MA; 10 mmol/L) or (C) hydroxychloroquine (HCQ; 8 μmol/L). Treatment with rapamycin (Rap; 0.25 μmol/L) served as positive controls. Lysate proteins were immunoprecipitated with anti-Beclin 1 antibody or control IgG (cIgG) and the precipitated proteins subjected to sequential immunoblotting with anti-Class III PI3K or anti-Bcl-2 antibodies.

Figure 4. Effects of autophagy inhibitors on doxorubicin- or melphalan-induced apoptosis. H929 or RPMI 8226 (A), newly diagnosed or refractory MM (B), or PBMC (C) cells were treated for 24 hours with doxorubicin (Dox) or melphalan (Mel) at the indicated concentrations in the presence or absence of 3-methyladenine (3-MA; 10 mmol/L) or hydroxychloroquine (HCQ; 8 μmol/L). The percentage of apoptotic cells was determined by flow cytometry using annexin V-Fluos assay and PI staining. Mean ± SD of three independent experiments. *P < 0.05.

Figure 5. Effects of doxorubicin or melphalan on Atg5 expression, and impacts of Beclin 1 or Atg5 knockdown on drug-induced LC3 processing or apoptosis. A, H929 and RPMI 8226 cells were treated for given times with (left) doxorubicin (Dox; 0.5 and 0.25 μmol/L, respectively) or (right) melphalan (Mel; 10 and 5 μmol/L, respectively). B and C, Indicated cells were infected with lentivirus producing both eGFP and short hairpin RNA (shRNA). Beclin 1 (B) or Atg5 (C) shRNA-expressing cells were collected by sorting of eGFP-positive cells and treated for 24 hours with Dox (left) or Mel (right) at the concentrations above. Protein lysates were subjected to immunoblotting with the indicated antibodies. D, Indicated cells functionally deficient in Beclin 1 or Atg5 were treated for 24 hours with Dox (top) or Mel (bottom) at the given concentrations. The percentage of apoptotic cells was determined by flow cytometry using annexin V-Fluos assay and PI staining. Mean ± SD of three independent experiments. *P < 0.05.
Figure 6. Combination of low doses of HCQ plus doxorubicin or melphalan inhibits plasmacytoma growth in CB-17/SCID mice. A and B, H929 tumors were established subcutaneously and treated with hydroxychloroquine (HCQ), doxorubicin (Dox), melphalan (Mel), or HCQ in combination with Dox or Mel. HCQ was injected i.p. at a dose of 60 mg/kg daily for 14 days; Mel was given i.p. at 3.5 mg/kg daily for 7 days; Dox was injected i.p. at 3 mg/kg on the first day of treatment, and then administered once more at 4-day interval. Tumor growth was monitored. Mean ± SD (n=9). *, P < 0.05, compared with vehicle control. Insets shows tumors resected from mice 30 days after treated with vehicle (control), or HCQ/Dox or HCQ/Mel combinations. C and D, Micrographs show apoptotic cells in tumors sectioned on day 30 (endpoint) from the mice under the treatment protocols given, as identified by a TUNEL assay (TUNEL-positive cells are dark brown) as well as H&E and Ki-67 staining. Shown photographs are representative of similar observations in 3 different mice receiving same treatment. TUNEL-positive cells were enumerated (in nonnecrotic areas of each section) at ×40 magnification for 5 fields for each tumor sampled. The average percentage of TUNEL-positive cells per field is reported as mean ± SD. Arrow: Immunostained tumor section of mice receiving the indicated combination treatment.
Fig 3
Fig 4
Fig 6
Targeting autophagy augments in vitro and in vivo antimyeloma activity of DNA-damaging chemotherapy

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