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Oncogenic transformation confers a selective susceptibility to the combined suppression of the proteasome and autophagy

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Abstract

The proteasome and the autophagy systems are two evolutionarily conserved mechanisms for degrading intracellular materials. They are functionally coupled and suppression of the proteasome promotes autophagy. While suppression of the proteasome leads to cell death, suppression of autophagy can be either pro-death or pro-survival. To understand the underlining mechanism of this dichotomy and its potential clinical implications, we treated various transformed and non-transformed human cells with proteasome inhibitors. We found that whether autophagy served a pro-survival role in this scenario was contingent on the cellular oncogenic status. Thus autophagy suppression enhanced apoptosis induced by proteasome inhibitors in transformed cells, but not in non-transformed cells. Oncogenic transformation enhanced the ability of cells to initiate autophagy in response to stress, reflecting a stronger dependence of transformed cells on autophagy for survival. Indeed, a combined use of Bortezomib, the only FDA-approved proteasome inhibitor for clinical use, and chloroquine, which inhibits autophagy by disturbing lysosomal functions, suppressed tumor growth more significantly than either agent alone in a xenograft model. These findings indicate that suppression of both intracellular degradation systems could constitute a novel strategy for enhanced cancer control in a tumor-specific way.

Keywords

macroautophagy; autophagy; proteasome inhibitors; apoptosis; cancer therapy

Introduction

The ubiquitin-proteasome system (UPS) is a major degradation system for short-lived proteins (1). Proteins to be degraded are labeled with ubiquitin. The ubiquitinated proteins are degraded by the 26S proteasome complex. The degradation is thus specifically targeted to a fraction of proteins. Prompt removal of these proteins is critical to the precise and timely regulation of intracellular signaling involved in multiple cellular processes, including cell proliferation and cell death. Macroautophagy (referred as autophagy hereafter) is another major intracellular degradation system. Unlike the UPS, autophagy is mainly responsible for the degradation of

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long-lived proteins and subcellular organelles (2). Autophagy plays important roles in development, cellular homeostasis and cell survival (3,4).

We had previously reported that the proteasome system and the autophagy system could be functionally coupled so that suppression of the former can activate autophagy via endoplasmic reticulum (ER) stress-mediated IRE-1 pathway (5). Consistently, ER stress could directly induce autophagy (6-8). Autophagy in this setting plays a compensatory role for the removal of misfolded proteins, thus mitigating ER stress. As the result, suppression of proteasome inhibitor-induced autophagy (5) or ER stress-induced autophagy resulted in enhanced cell death (8). Interestingly, this pro-survival activity of autophagy was notable in cancer cells but not in non-cancer cells (8). In the latter suppression of ER stress-induced autophagy did not promote cell death. It is not clear why and how autophagy could play different roles in regulating cell death. Indeed, autophagy could be pro-death as well in a number of cases (9).

The differential role of autophagy in regulating cell death in cancer cells vs normal cells could be explored for tumor-specific therapy. While direct ER stress inducers, such as thapsigargin or tunicamycin, have not been employed in clinics, proteasome inhibitors have been examined as a novel class of anti-cancer drug (10-12). Currently one proteasome inhibitor, Bortezomib (Velcade™), has been approved for treating refractory or relapsed multiple myeloma. There are clearly benefits in exploring the role of autophagy in proteasome inhibition for clinical application. On one hand, proteasome inhibitors have not been found to be effective in treating solid tumor in patients (13-15). On the other hand, resistance to proteasome inhibitors can develop in myeloma patients (12). Novel therapeutic strategies are much in need to combine proteasome inhibitors with other chemotherapeutic agents or radiation to overcome the resistance to or to broaden the therapeutic spectrum of the proteasome inhibitors (11,14,16).

In the present study we investigated the impact of autophagy on cell death induced by proteasome inhibitors in different types of cells, including paired human cell lines that differ in the transformation status. We demonstrated that inhibition of autophagy only enhanced cell death in the transformed but not in the non-transformed cells. Furthermore, combined suppression of the proteasome and autophagy was more effective than the suppression of either system alone in inhibiting tumor growth in a xenograft tumor model. These findings point out the importance of oncogenic status in how autophagy affects cell death, and indicate the benefits of suppressing both cellular degradation systems as a novel tumor-specific therapeutic strategy.

Materials and Methods

Reagents

The following antibodies were used: anti-Becn1 (BD Biosciences, San Diego, CA), anti- β -actin (Sigma, St. Louis), anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase-3 (Cell signaling, Danvers, MA) and HRP-labeled secondary antibodies (Jackson ImmunoResearch Lab, West Grove, PA). The rabbit polyclonal anti-Atg5 antibody was provided by Noboru Mizushima (17). The rabbit polyclonal anti-LC3B antibody was made using a peptide representing the N-terminal 14 amino acids of human LC3B and an additional cysteine (PSEKTFKQRRTFEQC). All chemicals were from Sigma, Invitrogen (Carlsbad, CA), or Calbiochem (Gibbstown, NJ).

Construct, siRNA and transfection

Adenovirus expressing GFP-LC3B (human) (Ad-GFP-LC3) was used as previously described (8). RNAi-mediated inhibition of gene expression was conducted by transfecting respective siRNA (0.24 μ M) into 1×10^6 cells using Oligofectamine (Invitrogen) for 48 hours before

analysis. The following siRNAs (Invitrogen) against human genes were used: *Beclin1* (5'-GGUCUAAGACGUCCAACAA-3') and LC3B (5'-GAAGGCGCUUACAGCUC AA-3'). A scrambled siRNA (5'-UUCUCCGAACGUGUCA CGU-3') (QIAGEN, Valencia, CA) was used as a negative control.

Cell culture

The colon cancer cell line, HCT116, was maintained in McCoy's 5A with the routine supplements (18). HCT116 cell line stably expressing GFP-LC3 had been described previously (5). The immortalized or transformed ovarian surface epithelial cell lines were generated as described previously by transfection and infection with SV40 T/t antigen, *hTERT* cDNA and *K-Ras*^{V12} (19). Immortalized sublines of T29 and T80 did not receive *K-Ras*^{V12}. These cells were maintained in the mixed Medium 199/MCDB105 medium (1:1) (Sigma) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). Their *K-Ras*^{V12} transformed derivatives, T29-K-Ras and T80-K-Ras were grown in the same medium supplemented with 10 ng/mL epidermal growth factor (EGF, BD Biosciences). Wild type and Atg5-deficient murine embryonic fibroblasts were immortalized using SV40 large T over-expression and cultured in DMEM with standard supplements (17). The human colon cell line CCD-18Co was purchased from ATCC (CRL-1459™) and cultured in DMEM with standard supplements. Normal human PBMC were purified using Histopaque density gradient medium with a standard protocol. All cultures were maintained in a 37°C incubator with 5% CO₂.

Microscopy

For fluorescence microscopy, cells were cultured in 12-well plate. Fluorescence images were acquired with an epi-fluorescence microscope (Nikon Eclipse TE200, Melville, NY) equipped with a SPOT RT SLIDER digital camera and the companion software (Diagnostics Instruments, Sterling Height, MI). For electron microscopy, tumor tissues were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), followed by 1% OsO₄. After dehydration, thin sections were cut and stained with uranyl acetate and lead citrate for observation under a JEM 1016CX electron microscope.

Analysis of cell death

General cell death was determined using propidium iodide staining (1 µg/mL). Apoptotic cells with condensed or fragmented nuclei were determined with Hoechst 33342 staining (5 µg/mL). Analysis of the effector caspase activity was performed as previously described (20) using Ac-DEVD-AFC as the substrate. TUNEL staining was performed as described previously (21).

Immunoblot assay

Cells were washed in PBS and lysed in RIPA buffer. Tumor tissues were suspended in RIPA buffer followed by sonication and centrifugation. Forty micrograms of protein was separated by SDS-PAGE and transferred to PVDF membranes. The membranes were stained with the indicated primary and secondary antibodies and developed with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Images were obtained using a Kodak Image Station 4000MM and analyzed by Kodak Image Software (Carestream Health, Inc., Rochester, NY).

Animal work

HCT116 cells were harvested and washed twice in PBS. Around 4×10^6 cells were resuspended in 200 µL PBS and inoculated on the right flank of each nude mice (6- to 8-week-old female BALB/c strain; Charles River). By day 14, tumors were well established in the mice with an average size around 300 mm³. Mice were then randomly assigned to 4 groups with 5 to 6 mice in each group. Mice were intraperitoneally given saline, Bortezomib (0.33 mg/kg body weight,

dissolved in saline with 0.3% DMSO), chloroquine (45 mg/kg body weight) or Bortezomib together with chloroquine. These agents were administrated every 3 days for a total of 6 times. Tumor growth was measured every two days after the first treatment and the volume of the tumor (mm^3) was determined using the formula, $\pi/6 \times A \times B^2$ (A is the larger diameter and B is the diameter perpendicular to A). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Statistical Analysis

Experimental data were subjected to z test, Student's t test, or one way analysis of variance analysis (ANOVA) with Scheffe's post hoc test where appropriate.

Results

Induction of autophagy by Bortezomib

Previously we had determined that a commonly used proteasome inhibitor, MG-132, could induce autophagy in HCT116, a colon cancer cell line and DU145, a prostate cancer cell line, which could be inhibited by knocking down essential autophagy genes or by a pharmacological agent, 3-MA (5). We verified and extended these findings with several other proteasome inhibitors, including Bortezomib, the only proteasome inhibitor currently approved by FDA for clinical use. Treatment of HCT116 with Bortezomib induced a dose-dependent increase in the endogenous LC3-II form (Fig. 1A) and in the exogenously introduced GFP-LC3II (Fig. 1B). In addition, GFP-LC3 became punctated, indicating a translocation to the autophagic membranes (Fig. 1C-D). The change in GFP-LC3 localization induction was not dependent on the expression of Bax, a key molecule known to contribute to the sensitivity of HCT116 cells to many types of cell death stimulations, including proteasome inhibitors (18,22).

To confirm that the accumulation of LC3-II and GFP-LC3 puncta was due to an increased induction of autophagy (23), but not to the blockage of the degradation of the exiting autophagosomes, we determined whether the autophagic flux was increased. We performed such an analysis based on the degradation of GFP-LC3. The autophagosomal GFP-LC3-II is degraded in the lysosome, but the GFP moiety is relatively resistant to hydrolysis. The appearance of the GFP moiety in cells could be used to indicate the breakdown of the autophagosomes (23). Basal autophagic activity in HCT116 cells stably expressing GFP-LC3 resulted in a low level of GFP-LC3 breakdown, which was significantly elevated following Bortezomib treatment (Fig. 1B). Furthermore, the accumulation of GFP moiety could be suppressed by the lysosomal protease inhibitors, E64D and pepstatin A, in parallel with a further accumulation of GFP-LC3-II as the result of the inhibition of the breakdown. The blockage of GFP-LC3 degradation led to a backup accumulation of GFP-LC3II (Fig. 1B) and the GFP-LC3 punctation (Fig. 1C-D). These observations thus indicated that inhibition of proteasome by Bortezomib indeed led to an elevated autophagic flux.

Combined inhibition of the proteasome and autophagy resulted in enhanced tumor cell death and suppression of tumor expansion

To determine whether the anti-tumor effects of proteasome inhibitors could be enhanced by the modulation of autophagy, we co-treated the HCT116 cells with Bortezomib and an autophagy inhibitor, 3-methyladenine (3-MA) or chloroquine (CQ). 3-MA can suppress the class III PI-3 kinase, which is required for the initiation of autophagy by many agents, including proteasome inhibitors (5,24). CQ can interfere with the lysosome pH level and thus can suppress lysosome-mediated degradation, which would lead to the inhibition of autophagy (23).

Indeed, such a combination led to an enhanced caspase-mediated apoptotic cell death, as indicated by the increased nuclear fragmentation and condensation and membrane permeability, which could be suppressed by z-VAD, a pan-caspase inhibitor (Fig. 2A&B). HCT116 cells with deficiency in Bax had comparable autophagy response to proteasome inhibitors (Fig. 1), but were relatively resistant to proteasome inhibitors (5, 22)(Fig. 2B) and other chemotherapeutic agents (18). However, these cells were not completely apoptosis-deficient, as they still express Bak, which could be activated under stronger apoptotic signals (25). Notably, suppression of autophagy in Bax-deficient HCT116 cells also significantly enhanced apoptotic death caused by Bortezomib (Fig. 2B). These findings were consistent with the notion that autophagy could play a compensatory mechanism to remove misfolded proteins and thus mitigate ER stress in the case of proteasome inhibition (5, 26). By suppression of autophagy, the ER stress level and therefore the magnitude of death stimulation would be elevated so that Bak could be readily activated (25).

To confirm the specificity of 3-MA and CQ in promoting apoptosis by suppressing autophagy, we transfected HCT116 cells with specific siRNA against the mammalian Atg6 homologue, *Beclin-1* or one of the mammalian Atg8 homologues, *LC3B*, two important molecules for autophagy. Immunoblot analysis indicated that both molecules could be effectively knocked down by the specific siRNAs but not a non-specific scrambled siRNA (Fig. 2C). Consistently, such treatment enhanced Bortezomib-induced apoptotic cell death (Fig. 2D&E). Moreover, we found that apoptosis induced by other types of proteasome inhibitors, such as lactacystin and ALLN, could be similarly enhanced (Fig. 2E), indicating the generality of the pro-survival function of autophagy in this setting.

The above finding also suggested that a simultaneous inhibition of autophagy may enhance the therapeutic efficacy of proteasome inhibitors. This could be potentially significant as proteasome inhibitors are currently only approved for the treatment of refractory or relapsed multiple myeloma and their therapeutic effects on other tumors have not been proven in the in vivo (13-15). We thus investigated whether suppression of autophagy could enhance the killing of solid tumors by Bortezomib in a xenograft model. HCT116 were inoculated to the lateral side of the right flank in nude mice. The average tumor volume in each mouse reached around 300 mm³ in 2 weeks. The mice were then treated with saline (as the control), Bortezomib, CQ, or Bortezomib plus CQ every 3 days for 6 times. Tumor progression was followed up by determining the tumor volumes every other day. CQ was chosen over 3-MA because of the comparable efficacy (Fig. 2A&B) and its FDA-approved status for clinic use.

Bortezomib alone retarded tumor growth, compared to the saline treatment (Fig. 3A). Interestingly, CQ alone also had inhibitory effects, suggesting that the basal autophagy activity was also beneficial for tumor growth. However, it was the combination of the Bortezomib and CQ that led to the most significant inhibition of tumor expansion, indicating that suppression of the proteasome together with the compensatory autophagy would cause the maximal stress and demise to cancer cells. Consistently the combined treatment induced a higher level of apoptosis, as indicated by the increased caspase activities and TUNEL staining in the tumor samples (Fig. 3B-C). The average body weight of mice in each group was around 21 grams, which was not significantly changed during the treatment.

Electron microscopic examination of the tumor samples indicated that there were indeed an increased number of autophagic vesicles following Bortezomib treatment (Fig. 3D-E). Consistently, the LC3-II form was also elevated (Fig. 3F). On the other hand, CQ caused the blockage of the degradation of autophagosomes, and therefore LC3-II, in the lysosome, resulting in an arrest of the autophagic flux and the accumulation of autophagic vesicles (Fig. 3D-E) and LC3-II (Fig. 3F). Combined use of Bortezomib and CQ led to a more significant manifestation of these changes, reflecting the effects on both the input and the output of the

autophagy flux. Overall, the status of autophagy in the tumor samples (Fig. 3D-F) recapitulated that of cultured cells (Fig. 1) upon the same type of treatment, indicating that the same signaling pathway was followed and targeted.

Inhibition of autophagy in normal or non-transformed cells did not enhance proteasome inhibitor-induced apoptosis

To determine the potential toxicity of the combined suppression of the proteasome and autophagy in normal cells, we first examined the impact of such treatment on freshly isolated normal human peripheral blood mononuclear cells (PBMC), commonly used for assessing toxic side effects of chemotherapy. We found that Bortezomib and other proteasome inhibitors could induce autophagy in PBMC, based on LC3-II formation, which could be suppressed by 3-MA (Fig. 4A and data not shown). Toxicity was low for these proteasome inhibitors, particularly for Bortezomib (11,12) (Fig. 4B-D). Notably, in contrast to what was observed in the cancer cells, suppression of autophagy with 3-MA did not enhance the toxicity of the proteasome inhibitors in normal PBMC.

We further confirmed the low toxicity of this combined treatment in a normal human colon cell line, CCD-18Co. Suppression of autophagy induced by proteasome inhibitors in this cell line by either 3-MA or siRNA-mediated knockdown of *Beclin 1* did not increase apoptosis caused by the same proteasome inhibitors, based on PI staining, apoptotic nuclear morphology or caspase activity (Fig. S1 and data not shown). Finally, we employed non-transformed but immortalized murine embryonic fibroblasts (MEFs), in which autophagy is suppressed by the genetic deletion of Atg5 (Fig. S2A). Consistently, apoptosis induced by proteasome inhibitors was no higher in Atg5-deficient cells than in the wild type cells (Fig. S2B-D). Similarly, when the wild type MEFs were co-treated with 3-MA and a proteasome inhibitor, there was also no increase in cell death, compared to the proteasome inhibitor alone (Data not shown). In fact, the toxicity of proteasome inhibitors in the MEFs could be reduced by the suppression of autophagy in these cells. These data thus indicate that inhibition of autophagy in the normal and/or non-transformed cells did not enhance the toxicity of proteasome inhibitors, which was contrary to the effect in the cancer cells.

Oncogenic transformation was correlated with a protective role of autophagy induced by proteasome inhibitors

To understand the potential mechanisms that might account for this differential role of autophagy in regulating cell death, we hypothesized that oncogenic transformation might be a key event that leads to such a difference. To explore this possibility, we adopted a recently developed cellular system that employed matched isogenic human ovarian epithelial cell lines (19). SV40 T/t-transfected ovarian epithelial cell lines (IOSE-29 and IOSE-80) were further infected with retrovirus expressing a full-length hTERT cDNA. Immortalized sublines (T-29 and T-80) were then transformed with the constitutively activated K-Ras^{V12} to generate the transformed T-29-K-Ras^{V12} and T-80-K-Ras^{V12}. Previous studies had shown that T-29-K-Ras and T-80-K-Ras cells exhibited neoplastic behaviors, capable of forming anchorage-independent foci in soft agar and tumors in nude mice, whereas their respective non-transformed counterparts, T-29 and T-80, did not exhibit any of these behaviors (19). Thus these cell lines were appropriate for the investigation of the role of oncogenic transformation in the autophagy regulation of cell death.

Bortezomib could readily induce autophagy in both the T-29 (Fig. 5) and the T-80 (Fig. S3) cells. Accumulation of GFP-LC3 puncta could be further increased by a concurrent treatment of CQ, indicating that there was a net increase of autophagy flux following proteasome inhibition. Interestingly, we observed that the transformed T-29-K-Ras and T-80-K-Ras cells seemed to have a significantly stronger autophagy response than their non-transformed T-29

and T-80 counterparts. This differential response did not seem to be limited in proteasome inhibition, as it could be also seen following amino acid starvation (Fig. S4). Furthermore, while CQ alone could induce an increase in GFP-LC3 puncta due to the blockage of basal autophagosome degradation, such an increase was even stronger in the transformed line than in the non-transformed lines, suggesting that there was an increased autophagy both at the basal level and following stressing in the transformed cells, and implying the impact of oncogenic transformation on autophagy response.

We then examined how autophagy might affect cell death in these cells. The non-transformed cells were much less sensitive than the K-Ras transformed cells to Bortezomib in both T-29 (Fig. 6) and T-80 (Fig. S5) cells as shown before (27), consistent with the observations made in other types of non-transformed cells (Fig. 4, Fig. S1-S2). More importantly, knockdown of the essential autophagy gene *Beclin-1* (Fig. 6A) or co-treatment with 3-MA (Fig. S5) greatly increased Bortezomib-induced apoptosis and caspase activation in the transformed cells but had little such effects in the non-transformed cells (Fig. 6B-C, Fig. S5). These results thus indicate that oncogenic transformation could be a key factor in determining how autophagy may affect cell death.

Discussion

Autophagy may play different roles in regulating apoptosis in cancer cells and in non-transformed cells under stress

Inhibition of autophagy promotes proteasome inhibitor-induced cell death in cancer cells, but not in primary cells or in non-transformed cell lines. This difference may not be due to the disparity in cell type, since it could be also observed in matched isogenic ovarian epithelial cell lines differing only in transformation status. It is not quite understood how autophagy may regulate cell death in different ways. Autophagy has been shown in earlier studies to be either cytoprotection (28-30) or cytotoxic (9,31) under various stress conditions. Although this difference may be related to the type of stress and the types of cell death being affected, a clear pattern has not emerged that could confer a clear mechanistic insight.

Our earlier studies and the present study together suggest that the oncogenic status of the cell is correlated with the effects of autophagy on cell death during the response to proteasome inhibitors or ER stress (5,8). It had been previously noted that normal cells were much less sensitive to the toxicity of proteasome inhibitors than the transformed cancer cells (11,12). The contribution of the oncogenic status rather than the cell type to this difference in death susceptibility could be further demonstrated with the use of the matched isogenic transformed and non-transformed ovarian epithelial cell lines (Figs. 6,S5)(27,32). Transformed cells may in general be more sensitive to stress and cell death, including that caused by misfolded proteins (16). Transformed cells may thus be more ready to mount any protective mechanisms, such as autophagy, and become more dependent on these mechanisms for survival. Indeed, a higher level of autophagy could be induced by proteasome inhibitors or starvation in the transformed cells than in the non-transformed cells (Figs. 5,S3, S4). In addition, autophagy is clearly protective in the setting of proteasome inhibition as it helps to reduce ER stress caused by misfolded proteins (5,8).

There could be multiple mechanisms for stronger autophagy responses in cancer cells. One possibility is related to reactive oxygen species (ROS). Oncogenic transformation of ovarian epithelial cells with *Ras*^{V12} has been reported to cause elevated generation of ROS, compared to their non-transformed counterparts (32). ROS have been implicated in the induction of proteins misfolding via oxidation (33), which can in turn promote autophagy for clearance. ROS may also have a more direct effect on autophagy induction via the regulation on Atg4B, which is required for LC3 processing, during starvation (34). These observations may help to

explain why proteasome inhibitors or starvation could induce a more robust autophagy response in transformed cells than in non-transformed cells. In contrast, autophagy may not be as critical in protecting against cell death in the non-transformed cells due to a lower level of stress. An unbalanced activation of autophagy may instead by itself be cytotoxic. These issues clearly need to be further addressed in future studies.

The combined use of autophagy inhibitors and proteasome inhibitors may prove to be an effective strategy for cancer control

Single use of proteasome inhibitors may not be effective for resistant myeloma and for solid tumors (12-15). While a number of combinatory approaches have been proposed (11,14,16), it seems that simultaneous inhibition of autophagy and proteasome function could offer several unique advantages based on this study and our earlier works (5,8). First, the combination aims at blocking both cellular degradation systems, which seem to be functionally coupled and complemented to each other. Second, suppression of autophagy enhances the cytotoxicity of proteasome inhibitors at an upstream level by elevating ER stress and the death stimulation, which may reduce the development of resistance. This is best illustrated by the enhanced killing of the Bax-deficient HCT116 cells (Fig. 2), which is otherwise quite resistant to Bortezomib despite the presence of Bak. Third, the combination could simultaneously target to the cell survival and cell death pathways. Finally, such a combination maintains the relative selectivity of proteasome inhibitors toward cancer cells with a low toxicity for normal cells.

The last point as demonstrated in the present study is particularly relevant and significant in cancer therapy, considering that several other regimes have been also examined that suppress autophagy to enhance cancer cell death induced by hypoxia (29), alkylating agents (35), or histone deacetylase inhibitors (36). Although the effect of these combinations on the survival of non-transformed normal cells has not been examined, it would be reasonable to speculate that the rationale defined in the present work may also be applicable in these scenarios, that is, the normal cells might be less affected by these combinations as well.

In the setting of animal studies and clinical trials, the use of chloroquine may be preferred due to its well defined pharmacological dynamics, being well tolerated and plentiful clinical experience in using this drug in other disease conditions. There could be foreseeable concerns with chloroquine, since it is not a specific suppressor for autophagy, but seems to disturb the lysosomal function in general. Whether a long-term use of this agent in the context of cancer therapy may elicit additional side effects is not known. However, in the current absence of any autophagy-specific inhibitors, chloroquine would be a reasonable candidate for use in combination with proteasome inhibitors in clinical trials to promote a better cancer control.

It has to be pointed out that suppressing autophagy is not a generic strategy to enhance therapeutic efficacy for all types of cancers. Depending on the types of cancer and the primary therapeutic agents, promoting autophagy may be instead necessary to enhance cancer cell death. In these cases, cytotoxic, rather than cytoprotective, effects, had been shown to be caused by autophagy (37).

Finally, the physiological role of autophagy in normal cells seems to be related to the control of metabolic stress and maintenance of genome and chromosomal stability (38). Autophagy may help to control intracellular ROS level and DNA abducts by removing damaged mitochondria (39). These effects may contribute to the control of tumorigenesis, as supported by the demonstration of the tumor suppressor role of Beclin 1 (40). While these observations do not necessarily contradict with those made in the context of cancer therapy regarding the function of autophagy, the impact of the long-term suppression of autophagy via pharmacological agents has yet to be defined.

In conclusion, we have provided evidence that oncogenic status may decide how autophagy affects cell death and that a combined use of proteasome inhibitors and autophagy inhibitors can selectively enhance cell death in the transformed cancer cells. This novel strategy may offer unique advantages in cancer control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Induction of autophagy by Bortezomib

(A). Bax-positive and Bax-negative HCT116 cells were treated with different doses of Bortezomib for 16 hours. Total lysates were prepared and subjected to immunoblot analysis. (B). Bax-negative HCT116 stably expressing GFP-LC3 were treated with vehicle control or Bortezomib (20 nM) in the presence or absence of E64D (10 μM) and pepstatin A (10 μM) for 16 hours. Total cell lysates were subjected to immunoblot analysis using an anti-GFP antibody. Digital data (mean ± SD) were from densitometry analysis of the GFP band from at least three independent experiments. (C-D). The same cells were treated with vehicle control (a), Bortezomib (20 nM, b), chloroquine (10 μM, d) or the combination of the two (c) for 16 hours and then examined by fluorescence microscopy (C). The numbers of GFP-LC3 dots per cell in each condition were quantified (D). Data (mean ± SD) are representative of at least three independent experiments. *: $p < 0.01$; #: $p < 0.05$ (one way ANOVA with Scheffe's post-hoc test).

A

B

Figure 2. Bortezomib-induced autophagy is cytoprotective

(A). Bax-positive HCT116 cells were either untreated (a) or treated with Bortezomib (20 nM) alone (b) or in the presence of 3-MA (10 mM, c), CQ (10 μ M, d), or z-VAD (50 μ M, e) for 24 hours. Cell death was determined by nuclear staining with Hoechst 33342 for apoptotic cells with fragmented or condensed or nuclei (arrows), and by propidium iodide staining for cytoplasmic membrane permeability change. (B) Bax-positive and Bax-negative HCT116 cells were treated as in panel A and the percentages of cells with defined changes were quantified. Upper panel: apoptotic cells with fragmented or condensed or nuclei; lower panel; propidium iodide staining positive cells. (C). Bax-deficient HCT116 cells were transfected with a negative siRNA (Neg) or Beclin-1 (Bec) or LC3B-specific siRNA (120 nM) for 48 h and analyzed by immunoblot with indicated antibodies. (D). siRNA-transfected cells were then treated with Bortezomib (20 nM) for another 24 hours. Cell death was determined as in panel A. a: Neg siRNA only; b: Neg siRNA plus Bortezomib; c: siRNA against Beclin 1 plus Bortezomib and d: siRNA against LC3B plus Bortezomib. Arrows indicate fragmented or condensed nuclei. (E). siRNA-transfected cells were treated with Lactacystin (5 μ M), ALLN (10 μ M) or Bortezomib (20 nM) for another 24 hours. Cell death was determined as in panel A. Data (mean $\pm$ SD) are representative of at least three independent experiments. *: $p < 0.01$ (z test).

D **E** **F**

Figure 3. Combined suppression of the proteasome and autophagy enhances the inhibition of tumor growth in vivo

(A). Balb/c nude mice were implanted with 4×10^6 Bax-positive HCT116 cells on the right flank. Fourteen days after inoculations, mice were grouped and intraperitoneally given saline, Bortezomib (Bort, 0.33 mg/kg), Bortezomib plus chloroquine (CQ, 45 mg/kg) or CQ alone every 3 days for 6 times as indicated by the arrows. The first treatment day is designated as day 0. Tumor volume (mm^3) was determined every two days after the first treatment (day 0) until day 16 and the mean tumor volumes were calculated ($n=5-6$ per group). *: $p<0.01$ (Bortezomib/CQ group versus the saline group, one way ANOVA with Scheffe's post-hoc test). (B). Total lysates were prepared from each tumor sample and pooled in equal amount of protein within each group. The lysates were then analyzed for effector caspase activities using Ac-DEVD-AFC as the substrate. Data (mean $\pm$ SD) of independent triplicate measurements were expressed as fold of increase over the saline control. (C). Tumor samples from each groups were subjected to TUNEL staining and the percentage of positive cells (mean $\pm$ SD) were determined *: $p<0.01$ (z test). (D). Electron microscopic examination of tumor samples recovered from nude mice treated for 16 days with saline (a), Bortezomib (b), Bortezomib+CQ (c), or CQ only (d). Arrows: autophagic vesicles; N: nuclei. Scale bar: 1 μm . (E). The number of autophagic vesicles per 100 μm^2 area was determined (means $\pm$ SD). *: $p<0.01$, #: $p<0.02$ (one way ANOVA with Scheffe's post-hoc test). (F). An equal amount of protein from each tumor sample within the same group ($n=5-6$) was combined and analyzed by immunoblot assay with anti-LC3 and anti- β -actin antibodies. Digital data (mean $\pm$ SD) were from densitometry analysis of the LC3-II band from at least three independent experiments.

Figure 4. Suppression of autophagy in normal human peripheral blood mononuclear cells does not enhance the toxicity of proteasome inhibitors

(A). Human PBMC were incubated with MG132 (0.5 μ M) or Bortezomib (20 nM) in the presence or absence of 3-MA (10 mM) for 24 hours. Immunoblot was then performed using the anti-LC3 antibody. (B-D). Human PBMC were treated with MG132 (0.5 μ M), ALLN (10 μ M) or Bortezomib (20 nM) in the presence or absence of 3-MA for 24 hours. Cell death was determined by propidium iodide staining (B) or by nuclear Hoechst 33342 staining for fragmented or condensed nuclei (C). Effector caspase activities were determined using Ac-DEVD-AFC as the substrate (D).

Figure 5. Transformed cells can initiate a more potent autophagic response than the matched non-transformed cells

(A). T-29 and T-29-K-Ras cells were first infected with Ad-GFP-LC3 for 24 hours and then treated with vehicle control, Bortezomib (20 nM), Bortezomib plus CQ (10 μ M) or CQ alone for 24 hours. Cells were then analyzed by fluorescence microscopy for LC3 translocation.

(B) The percentages (mean $\pm$ SD) of GFP-LC3-positive cells showing puncta were determined.

*: $p < 0.01$ (z test). (C) The numbers of GFP-LC3 puncta per cells (mean $\pm$ SE) were determined.

*: $p < 0.05$ (Student's t test).

Figure 6. Suppression of autophagy promotes proteasome inhibitor-induced cell death in transformed but not in matched non-transformed cells

(A). T-29 and T-29-K-Ras cells were first transfected with a negative siRNA (Neg) or Beclin-1 specific siRNA (120 nM) for 48 hours. The total lysates were analyzed by immunoblot for the expression of Beclin 1 and β -actin. (B-C). siRNA-transfected cells were then treated with Bortezomib (40 nM) for another 24 hours. Cell death was then determined by nuclear Hoechst 33342 staining for fragmented or condensed nuclei (B). *: $p < 0.01$ (z test). Effector caspase activities were measured using Ac-DEVD-AFC as the substrate (C). *: $p < 0.01$ (one way ANOVA with Scheffe's post-hoc test).