Abstract

**Purpose:** Autophagy consists of lysosome-dependent degradation of cytoplasmic contents sequestered by autophagic vesicles (AV). The role of autophagy in determining tumor aggressiveness and response to therapy in melanoma was investigated in this study.

**Experimental Design:** Autophagy was measured in tumor biopsies obtained from metastatic melanoma patients enrolled on a phase II trial of temozolomide and sorafenib and correlated to clinical outcome. These results were compared with autophagy measurements in aggressive and indolent melanoma cells grown in two- and three-dimensional (3D) culture and as xenograft tumors. The effects of autophagy inhibition with either hydroxychloroquine or inducible shRNA (short hairpin RNA) against the autophagy gene \( ATG5 \) were assessed in three-dimensional spheroids.

**Results:** Patients whose tumors had a high autophagic index were less likely to respond to treatment and had a shorter survival compared with those with a low autophagic index. Differences in autophagy were less evident in aggressive and indolent melanoma cells grown in monolayer culture. In contrast, autophagy was increased in aggressive compared with indolent melanoma xenograft tumors. This difference was recapitulated when aggressive and indolent melanoma cells were grown as spheroids. Autophagy inhibition with either hydroxychloroquine or inducible shRNA against \( ATG5 \) resulted in cell death in aggressive melanoma spheroids, and significantly augmented temozolomide-induced cell death.

**Conclusions:** Autophagy is a potential prognostic factor and therapeutic target in melanoma. Three dimensional culture mimics the tumor microenvironment better than monolayer culture and is an appropriate model for studying therapeutic combinations involving autophagy modulators. Autophagy inhibition should be tested clinically in patients with melanoma. *Clin Cancer Res; 17(10); 1–12. ©2011 AACR.*

Introduction

Although combination regimens involving cytotoxic chemotherapies or targeted therapies have improved survival in a number of malignancies, similar approaches have failed to improve survival in patients with metastatic melanoma (1). One common mechanism of resistance to chemotherapy and targeted therapies that has more recently been recognized is autophagy. Autophagy is a catabolic process characterized by the formation of autophagic vesicles (AV) that sequester damaged organelles and proteins and target them for degradation through fusion with the lysosomes (2). Autophagy is increased in cells faced with metabolic stresses including growth factor withdrawal (3), nutrient deprivation, and hypoxia (4, 5). Multiple cancer therapies, including cytotoxic chemotherapy, kinase inhibitors, proteasome inhibitors, radiation, and angiogenesis inhibitors can induce autophagy in most cancer cell lines (6). Although under certain experimental conditions, stress-induced autophagy can result in the death of cancer cells in traditional 2-dimensional (2D) culture, stress-induced autophagy contributes significantly to the survival of tumor cells growing within the tumor microenvironment (7). Besides metabolic or cell intrinsic stresses, therapy-induced autophagy can limit the antitumor efficacy of a number of therapies. Our previous work showed that cotreatment with the autophagy inhibitor hydroxychloroquine (HCQ) could effectively block the last step of autophagy and enhance cell death induced by activation of p53 or treatment with alkylating chemotherapy in a model of Myc-induced tumorigenesis (8, 9). Based on this finding and reports from other investigators that
The role of autophagy in determining the aggressiveness and therapeutic resistance of melanoma has not been fully appreciated. In this study, we define an objective index to enumerate autophagy levels in primary human melanoma tissues. A high autophagic index is associated with poor prognosis and resistance to therapy in patients with advanced melanoma. The high levels of autophagy observed in aggressive human tumors was reproduced only when melanoma cell lines were grown as three dimensional spheroids or xenograft tumors, and not when cells were grown in monolayer culture. The spheroid model showed that autophagy inhibition alone and in combination with chemotherapy selectively resulted in cell death in aggressive melanoma cells. The results of this study identify autophagy as a new therapeutic target in melanoma, and provide the preclinical rationale for investigating autophagy inhibitors in melanoma.

in clinical samples, and identifies a laboratory model system to study the implications of this finding.

Materials and Methods

Patient samples, tumor genotype, and clinical outcomes

Cutaneous tumor biopsies were obtained from metastatic lesions from stage IV melanoma patients with or without brain metastases enrolled on a 4-arm phase II trial of the oral alkylation chemotherapy temozolomide and oral multikinase inhibitor sorafenib (11). For details of study rationale, patient eligibility, schedule of treatment, assessments and trial results see the work of Amaravadi and colleagues (11). The study protocol was approved by the institutional review boards at the University of Pennsylvania and Dana Farber/Harvard Cancer Center. All patients provided informed consent for treatment and biopsies before enrollment. Biopsies were carried out on all patients that had cutaneous metastatic lesions that were safe to biopsy, using local anesthetics followed by a punch biopsy of lesions. Isolated tumor tissue was immediately fixed in electron microscopy (EM) fixative. Mutations in BRAF (exons 11, 15) and NRAS (exons 1, 2) were from archival tumor blocks or fresh tumor biopsy as described (12). Samples were tested for mutations in exons 1 to 9 of PTEN as published (13). Exons were amplified by PCR, treated with ExoSAP and processed for sequencing using the Applied Biosystems BigDye Terminator v1.1 Cycle Sequencing Kit. Unidirectional sequencing was carried out on an Applied Biosystems 3130xl automated sequencer. Sequence traces were analyzed with Mutation Surveyor v3.2 (www.softgenetics.com). DNA from a genomic control as well as the GenBank file for PTEN (NC_000010.10) was used as a reference during mutation detection. All exons were sequenced in both the forward and reverse directions. Multiplex ligation–dependent probe amplification (MLPA) was used to screen for copy number changes in PTEN as previously described (14). Progression-free survival (PFS) was defined as the interval of time since receiving first study drug to time of clinical or radiographic progression, or death due to any cause. Kaplan–Meier estimates of PFS and overall survival, 95% CI, and the Wilcoxin log-rank test, used to define hazard ratios were calculated using GraphPad Prism software.

Cell lines and plasmids

Melanoma cell lines A375P, WM3918, SKMEL28, and C8161 were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 50 μg/mL gentamicin, and 25 mmol/L HEPES in the presence of 5% CO₂ at 37°C. A375P GFP-LC3, C8161GFP-LC3 were generated as previously described (8). To generate C8161 tet-shControl cells, the pSingle-tTS plasmid vector encoding an shRNA (short hairpin RNA) directed against luciferase was transfected into C8161 cells and positive clones were selected with neomycin and limiting dilution (Clontech). To generate C8161 tet-shATG5 cell lines, upper and lower

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Translational Relevance

The role of autophagy in determining the aggressiveness and therapeutic resistance of melanoma has not been fully appreciated. In this study, we define an objective index to enumerate autophagy levels in primary human melanoma tissues. A high autophagic index is associated with poor prognosis and resistance to therapy in patients with advanced melanoma. The high levels of autophagy observed in aggressive human tumors was reproduced only when melanoma cell lines were grown as three dimensional spheroids or xenograft tumors, and not when cells were grown in monolayer culture. The spheroid model showed that autophagy inhibition alone and in combination with chemotherapy selectively resulted in cell death in aggressive melanoma cells. The results of this study identify autophagy as a new therapeutic target in melanoma, and provide the preclinical rationale for investigating autophagy inhibitors in melanoma.
strand oligonucleotides directed against ATG5 were synthesized following manufacturer’s instructions. Five plasmids encoding distinct hairpin sequences were generated initially. Cotransfection of C8161 with a combination of 3 plasmid vectors (sense strand: tet-shATG5 hp 3'-CCAGATATTTCTGGAATGGAAA; tet-shATG5hp4: CTTTCTATTCA-GAACGTGT; tet-shATG5 5'-CCTGAAACAGAATCCTTAA) yielded the highest efficiency of doxycycline-inducible knockdown.

In vitro melanoma invasion assay, GFP-LC3 imaging, and immunoblotting

The BD BioCoat Matrigel invasion chamber (BD Biosciences) was used according to the manufacturer’s protocol and as previously described (15, 16). Briefly, 100,000 cells were plated in the top well. After 72 hours, cells from both upper and lower chambers were counted by trypan blue exclusion. Invasion rate was calculated as number of lower chamber cells/(number of lower chamber + upper chamber cells). For GFP-LC3 imaging, A375P GFP-LC3 and C8161 GFP-LC3 cells were exposed to the indicated treatments and fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed 3 times and centrifuged onto slides. Fluorescence imaging was captured at 100× magnification on a Nikon Eclipse E800 fluorescent microscope. Cells were scored as punctate if they had more than 4 GFP-LC3 dots. For immunoblotting, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% sodium deoxycholine, 0.1% SDS, 1% Triton X-100, 10 mMol/L Tris at pH 8.0, 0.14 mMol/L NaCl), protease inhibitors (Roche Diagnostics) and phosphatase inhibitor cocktail (Sigma). Immunoblotting was carried out as previously described (8) using the following antibodies (1:1,000 or 1:2,500): LC3 (QCB biologicals), Atg5 (Sigma), P62/SQSTM1 (Santa Cruz), phosphor-Akt (Cell Signaling), phosphor-p70S6K (Cell Signaling) actin (Sigma). Band densities from Western blots were quantified using Adobe Photoshop CS4 Extended and ImageJ (NIH). All immunoblots presented and quantified are representative of experiments repeated on 3 separate occasions.

Tumor xenograft experiments

Approval for animal care and use for these experiments was provided by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. All experiments were carried out using 5-week-old athymic nude mice (NCr nu/nu) from US National Cancer Institute. Cultured cells of A375P and C8161 were, respectively, harvested in ice-cold PBS and expanded in vitro by subcutaneous injection into the flanks of mice (1 × 10⁶ cell/flank). Tumor size was measured twice a week using calipers, and tumor volume was calculated using the following formula: volume (mm³) = A (length) × B (width) × (A + B)/2. For each tumor, sections of visually viable tumor tissue were fixed in 10% formalin for preparation of paraffin-embedded sections and H&E (hematoxylin and eosin), and 2% glutaraldehyde for EM. Tumor lyases were achieved through manual agitation of remaining tumor tissue in RIPA buffer.

Electron microscopy

Tissue obtained from human and mouse tumors and 3D spheroids were fixed with 2% glutaraldehyde and stored at 4°C until embedding. Cells were postfixed with 2% osmium tetroxide, this was followed by an increasing gradient dehydration step using ethanol and propylene oxide. Cells were then embedded in LX-112 medium (Ladd), and sections were cut ultrathin (90 nm), placed on uncoated copper grids, and stained with 0.2% lead citrate and 1% uranyl acetate (3). Images were examined with a JEOL-1010 electron microscope (JEOL) at 80 kV. For quantification of viable cells using electron micrographs of tumor tissue, high-powered micrographs (6,000×–20,000×) of 20 to 25 single cells from multiple distinct low-powered fields in each tumor were obtained. AVs were scored by investigators who were blinded to aggressive or indolent descriptors. Morphological criteria for AV included (i) circularity; (ii) contrast with structures that were white or lighter than the cytoplasm; (iii) vesicles with contents; (iv) vesicles more than 200 nm in size; and (v) vesicles more than 200 nm interior to the plasma membrane. Vesicular structures with cristae characteristic of mitochondria in cross-section, or with electron dense pigment were excluded and counted as mitochondria and melanosomes, respectively. Counts were represented as box and whisker plots or mean ± SEM.

Three-dimensional culture

Melanoma spheroids were implanted in collagen using the liquid overlay technique previously described (17). Briefly, 5,000 cells/well were plated on agar (1.5% in PBS) and allowed to grow for 72 to 96 hours. Most cell lines plated at the correct density formed spheroids in the middle of the well. Collagen matrix was formed by adding 1.3% NaHCO₃ to collagen mix (10% 10× Eagle’s minimal essential medium, 1.7 mmol/L l-glutamine, 10% FBS, bovine collagen; Organogenesis Inc.). Spheroids were harvested and resuspended in collagen matrix and plated in 24-well plates and supplemented with RPMI 1640 containing 10% serum. All spheroid experiments were conducted in triplicate. Spheroids implanted in collagen were imaged by brightfield and fluorescence microscopy at 40×. Fraction of cells invading collagen = (total area – core area)/total area. Lysates were prepared from spheroids by treating spheroids implanted in collagen with collagenase and then lysing-collected spheroids in RIPA buffer. Cell death was assessed using the Live/Dead Assay (Molecular Probes) and fluorescent microscopy. Spheroid dimensions and quantification of percentage of dead cells was done using the lasso and magic wand tools in Adobe Photoshop Extended CS4. The % dead cells = integrated pixel density of Dead/(Live + Dead).
Results

A high autophagic index is associated with poor survival in metastatic melanoma

To assess differences in autophagy in melanoma tumors, pretreatment punch biopsies were carried out on cutaneous tumors from 12 patients enrolled on a phase II trial of temozolomide and sorafenib (11). Samples were processed for EM. Striking differences in the autophagic index (the number of AV per cell) were apparent in cells with intact nuclear and cytoplasmic membranes. Some tumors had a low autophagic index and displayed few AV per cell (Fig. 1A, Supplementary Fig. S1C) whereas other tumors had a high autophagic index where most cells were engorged with vesicles (Fig. 1B, Supplementary Figs. 1D and E). Using strict morphological criteria (see Material and Methods), the mean number of AV per cell was scored for each tumor. The 2 cellular structures which most resemble AV are mitochondria (circular organelle containing cristae; Supplementary Fig. 1A) and melanosomes (pigment containing vesicles; Supplementary Fig. 1B). These structures were excluded from scoring of AV.

To determine if specific oncogenic mutations could be responsible for the observed differences in autophagy in patient tumors, genomic sequencing of tumor DNA was carried out. Recurrent somatic mutations in Ras signaling (Fig. 1B, Supplementary Figs. 1D and E). Using strict morphological criteria (see Material and Methods), the mean number of AV per cell was scored for each tumor. The 2 cellular structures which most resemble AV are mitochondria (circular organelle containing cristae; Supplementary Fig. 1A) and melanosomes (pigment containing vesicles; Supplementary Fig. 1B). These structures were excluded from scoring of AV.

To determine if specific oncogenic mutations could be responsible for the observed differences in autophagy in patient tumors, genomic sequencing of tumor DNA was carried out. Recurrent somatic mutations in Ras signaling involving BRAF and NRAS (50% and 10% incidence in cutaneous melanoma, respectively) are currently being used to subcategorize melanoma patients and decide treatment options (18). Activation of MAPK signaling has previously been described to promote autophagy in model systems (19). There was no significant difference in the mean number of AV per cell in patients whose tumors harbored mutations in BRAF or NRAS who were wild type for both genes. To investigate the impact of the most common mutation in melanoma that impacts PI3K/AKT/mTOR signaling on AV number, the phosphatase and tensin homolog (PTEN) gene was interrogated for the presence of mutation or homozygous deletion by exon sequencing and MLPA. No mutations or homozygous deletions of PTEN were detected in the patient samples with adequate DNA (data not shown). To determine if a high or low autophagic index had any effect on clinical outcome, biopsied patients were separated into 2 groups based on their duration of PFS on temozolomide and sorafenib. Although the combination of temozolomide and sorafenib was found to have rates of 6-month PFS that met benchmarks to be considered an active regimen (20), the response rates and overall survival were not different than previously published response rates for temozolomide alone, and a randomized trial involving this regimen was not pursued (11). Patients with a median PFS of less than 2 months (who derived no benefit from this treatment) had tumors with a significantly higher number of AV per cell than tumors from patients with median PFS of 2 or more months who derived clinical benefit from this treatment [median 8.4 and 3.9 AV per cell, respectively (P = 0.01); Fig. 1C]. This analysis indicated that 6 AV per cell could be used as a meaningful cutoff to distinguish tumors with high (≥6 AV per cell) and low (<6 AV per cell) autophagic indices in patient tumors. Kaplan–Meier survival analysis indicated that the median survival of melanoma
patients treated with temozolomide and sorafenib whose tumors had a low-autophagic index was 8 months compared with a median survival of 2 months in patients whose tumors had a high autophagic index ($P = 0.038$). There were no significant differences between the 2 groups in other known baseline characteristics that are prognostic for survival in melanoma [age, stage, elevated LDH, ECOG performance status, sex, prior therapy, prior temozolomide, presence of brain metastases (Supplementary Table 1)]. Recent reports indicate that autophagy genes may play a role in melanosome production (21). There was no significant difference in the percentage of tumors that were melanotic in each of these 2 groups, indicating that autophagy levels vary independently of pigment production in melanoma tumors.

**Autophagy in aggressive and indolent melanoma cell lines grown as monolayers**

To determine if the clinical observation that a high autophagic index is associated with aggressive tumor behavior, markers of autophagy were investigated in well-characterized melanoma cell lines with varying degrees of invasion and metastases: C8161 (22) and SKMEL28 (23) are cell lines known to grow rapidly and invade when grown as subcutaneous xenograft tumors, whereas A375P (22) and WM3918 (Herlyn, unpublished data) are cell lines which are known to grow slowly and have no invasive potential in vivo. In traditional monolayer culture, the growth rate of C8161 was significantly higher than A375 cells. SKMEL28 and WM3918 were the slowest growing cells (Supplementary Fig. 2A). Measurement of invasive potential was carried out using the Boyden chamber invasion assay. C8161 and SKMEL28 were the most invasive, whereas A375P and WM3918 cells were less invasive. C8161 cells were more than 7-fold more invasive than the least invasive WM3918 cell line (Fig. 2A). Together these studies confirmed the grouping of aggressive (C8161 and SKMEL28) and indolent (A375P and WM3918) cell lines.

To determine if differences in tumor cell autophagy could be observed in aggressive versus indolent melanoma cell lines, markers of autophagy were investigated in well-characterized melanoma cell lines with varying degrees of invasion and metastases: C8161 (22) and SKMEL28 (23) are cell lines known to grow rapidly and invade when grown as subcutaneous xenograft tumors, whereas A375P (22) and WM3918 (Herlyn, unpublished data) are cell lines which are known to grow slowly and have no invasive potential in vivo. In traditional monolayer culture, the growth rate of C8161 was significantly higher than A375 cells. SKMEL28 and WM3918 were the slowest growing cells (Supplementary Fig. 2A). Measurement of invasive potential was carried out using the Boyden chamber invasion assay. C8161 and SKMEL28 were the most invasive, whereas A375P and WM3918 cells were less invasive. C8161 cells were more than 7-fold more invasive than the least invasive WM3918 cell line (Fig. 2A). Together these studies confirmed the grouping of aggressive (C8161 and SKMEL28) and indolent (A375P and WM3918) cell lines.

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cell lines in vitro, autophagy reporter cell lines C8161 GFP-LC3 cells and A375P GFP-LC3 cells were generated. LC3 is a cytoplasmic ubiquitin-like protein which is conjugated to lipids on the surface of AVs (24). The GFP-LC3 fusion protein produces a diffuse fluorescence in the absence of autophagy and a punctate fluorescence when AV accumulate. Although some differences in the percentage of autophagic cells could be observed in A375PGFP-LC3 and C8161GFP-LC3 cells, there was a high percentage of punctate cells in both cell lines (Fig. 2B). Pharmacological induction of autophagy with the mTOR inhibitor rapamycin or blockade of the lysosome with the autophagy inhibitor chloroquine (CQ) would in both cases lead to AV accumulation that can be visualized by the GFP-LC3 reporter. Treatment with CQ or rapamycin resulted in an accumulation of AV in both aggressive (C8161 GFP-LC3) and indolent (A375P GFP-LC3) cell lines. Treatment of indolent or aggressive cell lines with low micromolar concentrations of HCQ did not induce cell death (Supplementary Fig. 2B).

Immunoblotting against the autophagy markers LC3, ATG5, and p62 was carried out on the panel of 4 cell lines cultured in complete medium and melanoma cells exposed to growth factor and nutrient withdrawal (Fig. 2C). LC3 can be detected by immunoblotting as an unconjugated (LC3I) species, and conjugated to the surface of AVs (LC3II; ref. 24). ATG5 is a part of the ATG5-ATG12-Atg16 complex that takes part in assembling the AVs (2). P62 is a cytoplasmic docking protein that binds ubiquitinated proteins and traffics them to AV for degradation. P62 binds to LC3, and is itself degraded in AV, therefore p62 levels can reflect autophagic flux. High p62 levels have been associated with genotoxic stress and implicated directly in tumorigenesis (25). Although indolent melanoma cell lines had higher LC3II/LC3I ratio and lower p62 levels compared with aggressive cells, suggesting that indolent cell lines were more autophagic than aggressive cells, these differences were not significant. There was no difference in ATG5 levels between aggressive and indolent cell lines grown in complete medium. To accentuate differences in autophagy between aggressive and indolent melanoma cells, cells were grown under conditions of metabolic stress (serum and nutrient withdrawal). Under these growth conditions, the LC3II/LC3I ratio increased in both indolent and aggressive cell lines with no significant difference between the 2 groups (Fig. 2D). Similarly no significant differences were observed in levels of ATG5 or p62 as measured by fold change from baseline. These results indicate that no significant differences in autophagy markers were observed in aggressive and indolent melanoma cells grown in complete medium or in conditions that are known to induce autophagy. Because growth factor signaling can directly regulate autophagy through mTOR signaling, immunoblotting against phospho-Akt and phospho-p70s6K was carried out. No significant differences in the phosphorylation status of Akt or p70s6K was apparent between indolent and aggressive cell lines in either complete or serum/nutrient-free medium (data not shown). Taken together these results indicate that differences in autophagy are minimal in aggressive and indolent melanoma cell lines when they are grown in 2D cell culture.

Differences in autophagy in indolent and aggressive melanoma tumor xenografts

To investigate the differences in autophagy in indolent and aggressive melanomas in tumor xenografts, A375P and C8161 cells were implanted in the flanks of nude mice. After 33 days of growth, C8161 tumors were significantly larger than A375P tumors (181 ± 86 and 50 ± 7 mm³, respectively; Fig. 3A). Freshly harvested tumor tissue was analyzed by EM, and tumor cell lysates were prepared for immunoblotting. EM images were used to score AV per cell. The mean AV per cell was significantly increased in C8161 xenografts compared with A375P xenografts (median values 2.8 and 0.8, respectively; Fig. 3B). Examination of low and high magnification EM images of A375P (Fig. 3C) and C8161 (Fig. 3D) showed differences in autophagy in indolent and aggressive melanoma are accentuated in vivo within the tumor microenvironment. To confirm the differences in autophagy obtained by EM morphological criteria, tumor cell lysates from C8161 and A375P were subjected to immunoblotting against the autophagy markers LC3, p62, and ATG5 (Fig. 3E). In 3 separate tumors for each cell line, a consistent pattern of increased expression of ATG5, decreased levels of p62 and increased levels of LC3II were observed in aggressive C8161 tumor cell lysates compared with indolent A375P tumor cell lysates. Significant differences for LC3II/LC3I and ATG5 were observed between aggressive and indolent xenografts. These findings indicate that autophagy is significantly increased in aggressive compared with indolent melanoma tumor xenografts.

Autophagy in 3D spheroid culture

Herlyn and colleagues have pioneered 3D spheroid culture to study drug resistance in melanoma (17). Having established that differences in autophagy between aggressive and indolent melanomas are striking in patients, and in xenografts but less apparent in traditional 2D monolayer culture, 3D spheroids were generated for aggressive (C8161 and SKMEL28) and indolent (A375P and WMU3918) cell lines. Briefly, cells were grown into spheroids in complete medium on an agar surface and implanted into a collagen matrix. Although the growth of the central spheroid was not significantly different between indolent and aggressive spheroids at 24 or 48 hours (Supplementary Fig. 3A), the total spheroid area was much larger in aggressive spheroids compared with indolent spheroids (Supplementary Fig. 3B). The fraction of cells invading collagen was significantly increased in aggressive compared with indolent (mean ± SEM: 0.66 ± 0.13 and 0.12 ± 0.07, P = 0.004) melanoma spheroids 48 hours after implantation into collagen. After implantation in collagen for 48 hours, the fraction of cells invading collagen was 16-fold higher in C8161 than in the least invasive A375P cell line (Fig. 4A). Western blot analysis of lysates obtained from
spheroids grown for 48 hours in collagen showed increased LC3II/LC3I ratio, decreased p62, and significantly increased ATG5 in aggressive compared with indolent cells grown in 3D spheroids (Fig. 4B). This combination of markers is indicative of higher levels of autophagy observed in aggressive melanoma cells grown in 3D culture than indolent melanoma cells. These results more closely resemble levels of autophagy markers found in xenografts than levels of these markers in the same cell lines grown in 2D culture. To characterize the autophagic index further within intact spheroids, A375P and C8161GFP-LC3 cells were grown as spheroids. GFP-LC3 fluorescence was diffuse in A375P spheroids and punctate in C8161GFP-LC3 spheroids (Fig. 4C). EM of aggressive and indolent spheroids also showed a significant increase in the number of AV per cell in C8161 spheroids compared with A375P spheroids (Fig. 4D). To assess the functional importance of increased levels of autophagy observed in aggressive compared with indolent melanoma spheroids, spheroids were treated with PBS or a low dose of the autophagy inhibitor HCQ. Cell death was observed in spheroids generated from both aggressive cell lines and not in spheroids generated in indolent cell lines (Fig. 4E).

Autophagy inhibition enhances cell death in aggressive melanoma spheroids

To study the specific effects of autophagy inhibition on the survival of aggressive melanoma cells grown in 3D culture, stable cell subclones of aggressive C8161 melanoma cell line were generated that expressed a dox-inducible control short hairpin RNA (C8161 tet-shControl) or a dox-inducible shRNA directed against the essential autophagy gene ATG5 (C8161 tet-shATG5). In C8161 tet-shControl cells, no significant change in levels of ATG5, LC3I, or LC3II was observed (Fig. 5A). In C8161tet-shATG cells, doxycycline treatment (dox) resulted in a significant decline in ATG5 levels, resulting in a reduced accumulation of AV as measured by a dox-associated decline in LC3II/LC3I ratios confirmed effective autophagy inhibition in these cells. Inducible knockdown of ATG5 resulted in minor growth inhibition of C8161 tet-shATG cells grown in 2D culture (Supplementary Fig. 3C). To determine the effects of knockdown of ATG5 on cell survival in 3D culture, spheroids generated from C8161 tet-shControl and C8161tet-shATG5 cells were implanted in collagen. After 72 hours of dox, spheroids were assessed by Live/Dead assay. Dox-induced expression of control shRNA...
resulted in no cell death in C8161 tet-shControl cells. In C8161 tet-shATG5 spheroids dox-dependent expression of shATG5 was associated with a greater than 2-fold increase in cell death in the center of doxycycline-treated compared with doxycycline-untreated spheroids (Fig. 5B). To determine if the cell death associated with temozolomide, the cytotoxic chemotherapy commonly used as a single agent to treat melanoma, could be augmented with combined autophagy inhibition, C8161 tet-shATG5 spheroids were treated with DMSO or temozolomide in the presence or absence of knockdown of ATG5. Cell death was observed in the periphery of cells treated with DMSO or temozolomide, but when temozolomide was combined with knockdown of ATG5, increased cell death was also observed in the center of spheroids. Quantification of cell death found that knockdown of ATG5 significantly enhanced temozolomide-induced cell death in C8161tet-shATG5 cells (Fig. 5C). To determine if this result could be reproduced with pharmacological inhibition of autophagy spheroids were treated with HCQ alone and in combination with temozolomide. In 2D cell culture, high micromolar concentrations (100 μmol/L) of HCQ result in minimal toxicity to C8161 and additive cytotoxicity is achieved only when combining high concentrations of temozolomide (500 μmol/L) and HCQ (100 μmol/L; Supplementary Fig. 3D). In contrast nanomolar concentrations of HCQ can elicit cell death in C8161 3D spheroids (Supplementary Fig. 3E). Nanomolar concentrations of HCQ augmented the cytotoxicity of temozolomide in 3D culture (Fig. 5D) indicating that this combination is worthy of testing in patients.

Discussion

Autophagy is a degradative process that was originally designated as type II–programmed cell death (26).
Self-eating, if persistent, can lead to depletion of cellular components resulting in autophagic, apoptotic, or necrotic cell death (6). Autophagy can limit tumorigenesis through the clearance of damaged organelles and the mitigation of genotoxic stress (27). BECN1 (beclin 1), an essential autophagy gene has been described as a haploinsufficient tumor suppressor gene whose monoallelic deletion results in accelerated tumorigenesis in mouse models (28). All of these facts point to autophagy as a tumor suppressor mechanism, which implies that indolent tumors would be expected to have higher levels of autophagy than rapidly proliferating aggressive tumors. Despite this role in limiting tumor development, once tumors are established, increasing evidence indicates autophagy allows tumor cells to survive within the tumor microenvironment (5, 6, 29–31). Much of this evidence comes from transgenic mouse models and xenografts engineered from genetically defined cell lines. Measuring autophagy is often difficult, even in these model systems, and this has limited studies of autophagy in human tumors.

This is the first study to measure autophagy by EM in human tumors and correlate this measurement to clinical outcomes of PFS and overall survival in cancer patients. Cutaneous metastases from patients with melanoma enrolled on a phase II clinical trial provided an ideal set of tumor samples for this purpose, because tumors were not large necrotic masses, the tissue was freshly harvested, and was not required for clinical purposes. Moreover, as autophagy is a dynamic process, measurement of autophagy in freshly obtained tissue at the time of entry into the clinical trial offers a unique opportunity to link clinical outcomes with autophagy.

Figure 5. Autophagy inhibition in 3D melanoma spheroids. A, immunoblotting of lysates from C8161 tet-shControl and C8161 tet-shATG5 cells treated with or without doxycycline (Dox) for 72 hours. B, C8161 tet-shControl and C8161 tet-shATG5 spheroids (arrows) were treated with or without dox for 72 hours and analyzed by Live (green)/Dead (orange) assay. C, C8161 tet-shATG5 spheroids were treated with DMSO (–TMZ) or Temozolomide (+TMZ) 10 μmol/L with and without doxycycline treatment. Representative images are overlays of separately captured images of fluorescence microscopy for live, dead, and brightfield images of spheroid structure to better define the localization within spheroids of dead cells. D, fluorescence images of C8161 tet-shControl spheroids grown in 3D culture imaged with the Live/Dead assay and treated with DMSO (–HCQ) or Temozolomide 10 μmol/L in the absence or presence of HCQ 10 nmol/L for 72 hours; scale bar: 200 μm (B–D); *, P < 0.05.
clinical trial was more likely to be representative of the biology of the metastatic disease at the time of treatment than measurement of autophagy in archival tumor tissue.

The degree of heterogeneity that was found in tumor cell autophagy in melanoma patients was striking. Mounting evidence indicates genetic alterations in oncogenes and tumor suppressor genes can dictate autophagy levels in cancer cell lines (32, 33). The role of activated kinases within the MAPK pathway has previously been described to promote autophagy (19, 34). In this study, heterogeneity in autophagy levels was not explained by genotype of the BRAF and NRAS genes, key drivers of MAPK signaling, but a small and significant difference in autophagy levels between these common melanoma genotypes could be found if larger numbers of patients were included. The autophagic index (mean number of AV per cell) was significantly higher in patients that derived little or no clinical benefit from the combination of temozolomide and sorafenib. Patients who had stable disease or responded to therapy had low levels of autophagy in their tumors. A high autophagic index as determined by counting AV per cell could be indicative of increased autophagy induction, or of a blockade of clearance of AVs. Our finding that autophagy inhibition selectively targets aggressive melanoma cells that have increased AVs in 3D culture suggests that the high autophagic index observed in the tumors of patients with aggressive melanomas is an indication of increased autophagy induction in response to stress. These findings validate the emerging preclinical evidence in multiple models of malignancy that autophagy plays a critical role in resistance to chemotherapy and targeted therapy (10). The results of this study indicate that pretreatment levels of autophagy can predict resistance to therapy, but additional studies are necessary before counts of AVs by EM can be considered a predictive marker.

Kaplan–Meier survival analysis indicated a 4-fold increase in the length of median overall survival in patients with low levels of autophagy compared with patients with high levels of autophagy. This finding indicates that autophagy could be a prognostic marker in addition to a predictive marker in melanoma patients. A similar conclusion was made in a recent report that found elevated levels of beclin 1 predicted poor survival in patients with nasopharyngeal carcinoma (35). Further development of assays to measure autophagy in human tissue that correlates well with the gold standard of EM are necessary to measure autophagy in larger numbers of patients using paraffin-embedded tissue. One limitation of this study is that autophagy was measured by EM in a small portion of one cutaneous tumor from each patient. Intratumoral variation in autophagy levels due to the geography of the local tumor microenvironment and intertumor variation in autophagy levels within the same patient were not addressed in this study. Emerging immunohistochemical autophagy markers will shed light on the variability of autophagy levels within one or many lesions in the same patient, and this degree of variability will be critical to determine before autophagy markers become candidates for prognostic or predictive biomarkers for melanoma.

To determine if the high-autophagic index found in aggressive melanomas and low-autophagic index found in indolent melanomas could be modeled in the laboratory, multiple markers of autophagy were measured in 4 melanoma cell lines with known degrees of invasiveness grown in traditional 2D culture. No clear differences between indolent and aggressive cell lines were observed in the well-established autophagy markers LC3 ATG5, or p62. Both aggressive and indolent cell lines increased autophagy equally in response to metabolic or therapeutic stress. In contrast, when grown as xenografts, aggressive melanoma cells had an increased autophagic index (along with increased LC3II/LC3I ratio and ATG5 levels and decreased p62 levels) compared with indolent xenografts recapitulating the findings made in human tumors. These results underscore the emerging appreciation for how the tumor microenvironment influences tumor cell autophagy, resulting in markedly different phenotypes when cells are grown in monolayer tissue culture or when they are grown in more complex microenvironments. Lu and colleagues reported experiments which found scheduled induction of autophagy produced cell death in 2D culture, but contributed to autophagic cell survival when the same cell line is grown as a xenograft (7). In this ovarian cancer model, the presence of cytokines (e.g., IGF-1), angiogenic factors (e.g., VEGF), and components of the extracellular matrix (e.g., collagen) all contribute to switch the cell fate from death to survival in autophagic cells.

Our previous work on the microenvironment in melanoma tumors suggest that the increased autophagy observed in aggressive compared with indolent melanomas, may be driven by nutrient limitation or hypoxia which in turn leads to the production of reactive oxygen species (ROS). Cryogenic NADH/flavoprotein fluorescence imaging (mitochondrial redox scanning) indicated that C8161-contained tumors were highly oxidized, characteristic of tissue starved of mitochondrial substrates, whereas A375P melanoma xenografts were uniform in a reduced state (36, 37). Imaging studies showed that the blood transfer rate (a surrogate of oxygen delivery) and capillary patency was significantly higher in A375P melanomas than in C8161 melanomas (36, 38). Thus, melanoma cells in the aggressive tumors appeared to be undergoing starvation of nutrients. Despite this metabolic challenge, highly metastatic melanoma tumor cores contained few apoptotic cells whereas A375P melanoma xenografts were uniform in a reduced state (36, 37). Imaging studies showed that the blood transfer rate (a surrogate of oxygen delivery) and capillary patency was significantly higher in A375P melanomas than in C8161 melanomas (36, 38). Thus, melanoma cells in the aggressive tumors appeared to be undergoing starvation of nutrients. Despite this metabolic challenge, highly metastatic melanoma tumor cores contained few apoptotic cells whereas A375P melanoma tumors contained a significantly higher number of apoptotic cells (38). The findings of the current study along with previous work characterizing the tumor microenvironment indicates that metabolic stress-induced autophagy in aggressive melanoma cells growing within the tumor microenvironment contributes to tumor cell survival and resistance to therapy, and therefore should be a target for novel therapies.

To develop a high throughput model to study autophagy and autophagy inhibition in melanoma further, the 3D spheroid model described by Herlyn and colleagues was
employed. As observed in the xenograft tumors, aggressive melanoma spheroids had higher LC3II/LC3I, and ATG5 levels and lower p62 levels than indolent melanoma spheroids. High levels of the essential autophagy protein ATG5 were detected in aggressive melanoma cells grown in spheroids and in tumor xenografts but not in the same cells grown in monolayer culture. Further studies are underway to understand the mechanism behind this differential expression and increased reliance on autophagy for survival in aggressive compared with indolent melanomas. Treatment with clinically achievable concentrations of the autophagy inhibitor HCQ, or knockdown of the essential autophagy gene ATG5 resulted in cell death in the center of aggressive but not indolent melanoma spheroids, indicating tumor cell autophagy in aggressive melanoma spheroids was promoting cell survival. Autophagy inhibition also augmented temozolomide-induced cell death in 3D spheroids showing how this model can be used to screen for drugs whose activity might be augmented by autophagy inhibition and provide the rationale for further preclinical or clinical trials. Based on these and other compelling preclinical studies (39, 40), a phase I trial of temozolomide and HCQ has been launched in patients with advanced solid tumors and melanoma (NCT00714181). As the number of potential autophagy inhibitors and the number of drugs that may be limited by autophagy increase, so does the need to identify patients that are more or less susceptible to this strategy. This study indicates patients with aggressive melanoma are more likely to have higher levels of autophagy in their tumor and therefore by more susceptible to autophagy inhibition as a therapeutic strategy. Additional studies will be necessary to determine if EM or other emerging biomarkers of autophagy such as ATG5 might be employed as prognostic and/or predictive biomarkers to serve this purpose.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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