Title

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Journal

Cancer Research, 72(17)

ISSN

0008-5472

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Publication Date

2012-09-01

DOI

10.1158/0008-5472.can-12-1076

Peer reviewed

Published in final edited form as:
Cancer Res. 2012 September 1; 72(17): 4294–4299. doi:10.1158/0008-5472.CAN-12-1076.

Tumor cell autophagy as an adaptive response mediating resistance to treatments like anti-angiogenic therapy

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Abstract

Autophagy is a lysosomal degradation pathway that can sequester cytosolic material including organelles nonspecifically in a process called nonselective macroautophagy, or can target specific protein aggregates designated for destruction in a process called selective autophagy. Autophagy is one mechanism that enables tumor cells to survive stressors in the tumor microenvironment, as well as injuries caused by treatments like chemotherapy or radiation therapy. The complexity of the role of autophagy in cancer is underscored by evidence that autophagy can allow premalignant cells to escape the genotoxic stress and inflammation that promote tumorigenesis, and by evidence that some tumor cells exhibit loss of autophagy capacity altogether through molecular mechanisms that have not yet been defined. Efforts to understand and modulate the autophagy pathway will be crucial to maximize the full therapeutic potential of cancer therapies which are currently hindered by tumor cell autophagy as a resistance mechanism.

Keywords

autophagy; glioblastoma; angiogenesis; hypoxia

INTRODUCTION

Cellular stressors activate autophagy, a pathway in which double membrane vesicles form and engulf damaged protein aggregates and organelles that are then delivered to lysosomes for degradation. Recent evidence suggests that, while autophagy may initially prevent tumor formation and growth, tumor cells respond to many treatment-related stressors by using autophagy as a cytoprotective mechanism leading to treatment resistance. Here, we review the key mediators of autophagy, the role of autophagy in tumor cell biology, evidence suggesting that autophagy can promote therapeutic resistance, and the challenges associated with using autophagy inhibition as a therapeutic strategy.

OVERVIEW OF AUTOPHAGY

Mammalian autophagy involves four steps: (i) formation of the phagopore (also called the isolation membrane) from the endoplasmic reticulum; (ii) assembly of autophagy-mediating proteins at the phagopore; (iii) engulfment of the phagopore by the endoplasmic reticulum to form double-membrane autophagosomes; and (iv) autophagosomes form mature degradative

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No conflicts of interest to report.

vacuoles (autolysosomes) by fusion with lysosomes. In terms of specific mediators of these four steps:

1. phagopore formation starts with ATG1, ATG13, and ATG17 forming a complex which recruits membrane protein ATG9 to the developing phagopore. Phagopore formation is aided by a class III phosphatidylinositol-3 kinase (PI-3KIII) Vps34 (vesicular protein sorting 34) and its binding partner Beclin-1 (ATG6), while the initial step of mitophagy, the autophagic degradation of mitochondria, is mediated by Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) (1). BNIP3 binds to Bcl2 and releases Beclin-1 from Bcl2 to initiate the Beclin-1-ATG14-PI3KIII complex.
2. proteins assembling at the phagopore include ATG7, which activates ATG12, enabling it to be transferred to ATG10, a carrier protein that potentiates covalent linkage of ATG12 to ATG5. The formation of the ATG12-ATG5 conjugate promotes the elongation and closure of the phagopore to form the autophagosome.
3. autophagosome formation involves the following steps: (i) ATG4-mediated cleavage of cytosolic microtubule-associated protein light chain 3 (LC3; mammalian homologue of ATG8), generating LC3-I; (ii) activation of LC3-I by ATG7; (iii) activated LC3-I, is transferred to ATG3, which conjugates LC3-I with phosphatidylethanolamine (PE) to generate LC3-II; and (iv) LC3-II is incorporated into the phagopore membrane, where it promotes fusion of the phagopore membrane to the endoplasmic reticulum by acting as a receptor for adaptor molecules on target membranes such as proteins and organelles marked for degradation. One of these adaptor molecules that binds LC3-II is p62/SQSTM1, which binds to ubiquitinated proteins and promotes turnover of p62-ubiquitinated protein aggregates.
4. Autophagosomes form mature autolysosomes by fusion with lysosomes - This aspect of autophagy is less well studied but requires G protein Rab7 in its GTP-bound state.

While autophagosomes can sequester cytosolic material including organelles nonspecifically in a process called nonselective macroautophagy, there is also evidence that a process of selective autophagy can occur as well. During selective autophagy, autophagic degradation of specific protein aggregates occurs (Figure 1). Selective autophagy is associated with degradation of p62 (2), a protein complex that binds ubiquitinated protein aggregates to target them for degradation. In contrast, nonselective autophagy involves (i) BNIP3, a hypoxia-inducible factor-1α (HIF-1α) downstream target gene and marker of autophagic destruction of mitochondria; and (ii) LC3, which, after conversion from its LC3-I form to its LC3-II form, is degraded by lysosomal enzymes in autolysosomes, causing the total amount of LC3 (LC3-I plus LC3-II) to drop (3).

Because autophagosomes were initially noted to accumulate in dying cells, the term “autophagic cell death” was created to describe a mode of cell death lacking features of apoptosis and instead marked by the cytoplasmic accumulation of autophagosomes. However, subsequent studies have shown that autophagy also can be activated by stressed cells to survive stressors by removing damaged proteins and organelles (4-6).

ROLE OF AUTOPHAGY IN CANCER

It has been hypothesized that autophagy protects cells from the genotoxic stress that can lead to oncogenic transformation by killing cells before DNA damage can be sustained. However, once this barrier has been overcome and a tumor has formed, some have hypothesized that the tumor will utilize autophagy as a survival mechanism to overcome the

stresses imposed during cancer progression and those caused by radiation or chemotherapy. When these stressors reach a critical point, autophagy is hypothesized to revert to mediating cell death.

Consistent with this hypothesis, a cytoprotective role of autophagy in established tumors exposed to stressors like anticancer treatments is suggested in studies where autophagy inhibitors like hydroxychloroquine or 3-methyladenine (3-MA) sensitize cancer cells to treatments like tamoxifen treatment (7), radiation (8), DNA alkylating agents cylophosphamide (7) and cisplatin (9), and tyrosine kinase receptor inhibitor imatinib (10). In contrast, some studies have suggested the converse, that autophagy can be associated with cell death in tumor cells treated with chemotherapy. For example, one report found that glioblastoma cells treated with dasatinib and temozolomide exhibited increased autophagy and increased cell death, although definitive correlation between the two phenomenon was not offered (11). Another report in which glioblastoma cells treated with the pan-Bcl-2 inhibitor (-)-gossypol exhibited increased autophagy and increased cell death carried the observation one step further by showing increased cell survival after (-)-gossypol treatment of cells transduced with shRNA targeting beclin1 or ATG5 (12). In murine colon carcinoma cells treated with methotrexate or oxaliplatin, autophagy was not required for chemotherapy-induced cell death but was required for the immunogenicity of dead cells by promoting release of ATP from dying cells (13).

It is possible that the differences in studies associating autophagy with tumor cell survival versus tumor cell death in response to therapies may merely reflect the extent of damage induced. Damage below the threshold of tolerance may allow autophagy to be associated with tumor cell survival, while damage beyond the threshold of tolerance may cause autophagy to promote tumor cell death. Ultimately, further work will be needed to clarify mechanisms rendering autophagy protective versus cytotoxic, including explanations for the molecular basis for variability in the role of autophagy in different cell types (14, 15).

The variable role in autophagy between different tumors could reflect some tumor cells exhibiting loss of autophagy function through as yet unidentified mechanisms that will likely include interactions between tumor suppressor and promoting genes and autophagy:

1. mTOR pathway negatively regulates autophagy - A major, and perhaps primary, regulator of autophagy is the mammalian target of rapamycin (mTOR) pathway. mTOR is activated downstream of PI3K-AKT, a pathway that is commonly dysregulated in human cancer. Activation of mTOR can also occur due to loss of tumor suppressors (LKB1, PML, PTEN, and TSC1/2) or through gain-of-function mutations in receptor tyrosine kinases (16). mTOR negatively regulates autophagy by causing phosphorylation of ATG13, which inhibits formation of a trimeric complex required for autophagosome formation (17). Because mTOR suppresses autophagy, normal liver cells deficient in PTEN, a tumor suppressor that inhibits mTOR by way of PI3-AKT inhibition, exhibit suppressed formation and maturation of autophagosomses (18). Similarly, when the Akt oncogene, an activator of mTOR, is inhibited by shRNA in a prostate cancer cell line, autophagy is promoted (19).
2. EGFR/Ras/MAPK pathway promotes autophagy - Murine embryonic fibroblasts (MEFs) with oncogene-mediated overactivation of Ras exhibit increased autophagy due to increased p62 expression (20). The effects of Ras overactivation on autophagy are important because the epidermal growth factor receptor (EGFR)/ Ras/mitogen-activated protein kinase (MAPK) signaling pathway is altered in several tumors, including over 40% of glioblastomas (21).

3. p53 mutations promote autophagy if localized to the cytoplasm - *p53* mutant proteins that localize to the cytoplasm of colon cancer cells promote autophagy(22). Mutations in the DNA binding regions of p53 did not affect autophagy, suggesting that as yet unidentified molecular features of p53 account for its autophagy suppression.

In cancer, hypoxia is a crucial cellular stressor faced by proliferating cells growing in a microenvironment containing abnormal vessels that fail to effectively deliver a blood supply to the tumor. Recent reports suggest that the cellular stress of hypoxia activates autophagy. Pathways activated by the tumor cell response to hypoxia that have been shown to contribute to autophagy include those mediated by hypoxiainducible factor-1α (HIF-1α, which is activated during physiological hypoxia (0.1%-3% O_2), or by HIF-1α-independent 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is activated during anoxia (≤ 0.01% O_2) and acts as an ubiquitous sensor of cellular energy status by responding to an ATP-depleted adenine nucleotide pool by phosphorylating many target proteins with functions related to energy metabolism (23).

Several intermediate factors have been shown to allow HIF-1α and AMPK to upregulate autophagy (Figure 1). As mentioned above, HIF-1α upregulates expression of BNIP3, a marker of mitophagy that is essential to hypoxia-induced autophagy (24). The upregulation of BNIP3 by hypoxia is particularly intriguing given the role of BNIP3 in mitophagy, which could be particularly important in allowing cells to adapt to hypoxia as the buildup of reactive oxygen species (ROS) in the mitochondria of hypoxic cells has been suggested to be a source of cell death. The importance of BNIP3 in the ability of tumor cells to overcome the hypoxia present in their microenvironment is suggested by the observed correlation of immunostaining for BNIP3 with poor survival in lung (25) and endometrial (26) cancers. The AMPK pathway activation increases tumor cell autophagy indirectly through inactivation of the mTOR complex (27), and directly through stimulating ULK1 (28) (Figure 1). The ability of tumor cells to use these two pathways to harness autophagy, and mitophagy in particular, as a survival promoting mechanism in hypoxia could be a valuable biologic mechanism supporting tumor growth and therapeutic resistance.

AUTOPHAGY AS AN ADAPTIVE RESPONSE TO CANCER TREATMENTS

Several cancer therapies, including DNA-damaging chemotherapeutic temozolomide (29) and radiation (8) induce autophagy in culture and animal models (30), and the autophagic response to many of these treatments is cytoprotective (31). Radiation therapy promotes autophagy by upregulating transcription of autophagy mediators Beclin-1, ATG3, ATG4, ATG5, and ATG12, with a survival-promoting effect confirmed by autophagy inhibition (8). Other studies have shown that some chemotherapy agents like histone deacetylase (HDAC) inhibitors (31) and cisplatin (32) induce autophagy by increasing production of ROS in mitochondria.

These observations reflecting autophagy as an adaptive response to radiation therapy and conventional DNA damaging chemotherapy have been augmented by our recent finding that autophagy is an adaptive response to a different type of therapy, anti-angiogenic treatment (33), whose ability to curb tumor progression by targeting abnormal tumor vessels has been confirmed by preclinical evidence and clinical trials (34). However, these initial successes were tempered by the failure of angiogenesis inhibitors to produce enduring clinical responses. For example, in phase II clinical trials of vascular endothelial growth factor (VEGF) neutralizing antibody bevacizumab in glioblastoma (GBM), 40-60% of tumors progressed after initially successful treatment (35), consistent with the development of resistance to anti-angiogenic therapy, a state exhibiting a poor prognosis and poor response to available treatments (36). We found that hypoxia increases after the devascularization

caused by anti-angiogenic therapy, consistent with the goals of these therapies, but that some tumor cells survive the hypoxic insult elicited by anti-angiogenic therapy through autophagy by activating the AMPK and HIF-1α pathways (33).

Our finding of hypoxia-induced autophagy in tumor cells as an adaptive response to the hypoxia caused by anti-angiogenic therapy can be expanded to determine the effect of hypoxia on cells in the tumor microenvironment. For example, we have found hypoxia does not induce autophagy in endothelial cells isolated from GBMs (unpublished data), consistent with our finding that the vessel density in GBMs resistant to anti-angiogenic therapy was suppressed (33) and suggesting that tumors grow during anti-angiogenic therapy without increased endothelial survival. Furthermore, because hypoxia increases the size of the cancer stem cell (CSC) population (37), one could hypothesize that hypoxia promotes autophagy in CSCs. Confirming this hypothesis would provide additional rationale for autophagy inhibition to prevent resistance to anti-angiogenic treatment.

The adaptive response of tumors to anti-angiogenic therapy may involve increased tumor cell invasiveness (38). Additional studies will be needed to determine whether cells surviving anti-angiogenic therapy through autophagy exhibit increased invasiveness, as occurs in cells treated with a chemical that induces autophagy (39). Demonstration that cells surviving anti-angiogenic therapy through autophagy exhibit increased invasiveness would suggest that autophagy inhibition could inhibit the invasion occurring after anti-angiogenic therapy by disrupting it at an earlier stage, which may be more effective than targeting invasion directly, as the numerous mediators of invasion make invasion difficult to pharmacologically disrupt.

AUTOPHAGY INHIBITION IN CANCER

Based on the preclinical evidence above, autophagy inhibition is currently being investigated as a way of modulating the response to cancer therapies in patients. Currently, the only FDA approved agents able to inhibit autophagy are chloroquine, an anti-malaria drug, and its derivative hydroxychloroquine, which block autophagy by disrupting lysosome acidification. One notable completed study was a randomized trial combining chloroquine with conventional treatment, defined as radiation plus temozolomide, for glioblastoma. The median survival was 24 months with chloroquine treatment versus 11 months without chloroquine treatment, a difference that was not quite statistically significant (40). The lack of statistical significance in that trial could mean that the effect is real but there was insufficient sample size (30 patients total) to achieve statistical significance or could mean that the observed difference was due to chance and that there really isn't an effect when combining chloroquine-mediated autophagy inhibition with standard glioblastoma treatment. If the latter were true it could be because (i) chloroquine failed to sufficiently inhibit autophagy in patients; or (ii) the role of autophagy in the temozolomide response might not be cytoprotective, as suggested by preclinical evidence (29).

There are currently 22 phase I/II cancer clinical trials involving chloroquine or hydroxyl-chloroquine open nationwide (www.clinicaltrials.gov), including two combining hydroxyl-chloroquine with bevacizumab and conventional DNA damaging chemotherapy, results of which could support the preclinical data we obtained showing a role for autophagy in resistance to anti-angiogenic therapy.

Despite these ongoing clinical efforts, the use of autophagy inhibition as a therapeutic strategy in cancer may need further preclinical evaluation to optimize the chances of success. Challenges in using autophagy inhibition as a therapeutic strategy are those described above in defining the biology of autophagy in cancer: (i) recognizing the dual roles for autophagy in tumors – cytoprotective or cytocidal depending on whether the tumor

is in early or late stages of oncogenesis or the type of tumor (41); and (ii) recognizing functional autophagy status in tumors, as some tumors may have defects in the autophagy pathway (13), while others will have preserved capacity for autophagy. The first challenge leads most observers to suggest that autophagy inhibition will be ineffective as monotherapy as basal autophagy may be cytocidal, while stress-induced autophagy as seen in response to traditional chemotherapy may be cytoprotective, a resistance response that can then be targeted when autophagy inhibition is combined with the chemotherapy. The second challenge suggests that biomarkers for the autophagy capability of individual tumors will be needed in order to identify tumors best served by a therapeutic strategy of autophagy inhibition.

Based on the hypothesis that tumor cells exhibit minimal basal survival-promoting autophagy and that autophagy may be most significant as an adaptive response to anticancer therapies, it is felt that autophagy inhibition will likely be of minimal utility as monotherapy. Therefore, the clinical trials of chloroquine and hydroxyl-chloroquine to date have all combined these agents with treatments which induce autophagy as an adaptive responsive.

Additional preclinical work will also be needed to develop autophagy inhibitors beyond chloroquine or hydroxyl-chloroquine. While preclinical studies like ours have suggested that these agents disrupt autophagy in animal models, other studies have shown that the ability of chloroquine to potentiate the effects of chemotherapies that induce autophagy may occur independent of autophagy disruption (42). Furthermore, it has yet to be proven that chloroquine or hydroxyl-chloroquine effectively block autophagy in human tumors or how the genetic makeup of these tumors influences their susceptibility to these agents. Should chloroquine or hydroxychloroquine ultimately prove to be too non-specific for clinical use as autophagy inhibitors, the development of more specific autophagy inhibitors will require focusing on kinases like ATG1or Vps34 or proteases like ATG4 that specifically regulate the activation of autophagy and autophagosome formation, with minimal intracellular roles outside of autophagy.

CONCLUSION

Autophagy is a lysosomal degradation pathway in which double membrane vesicles form and engulf damaged protein aggregates and organelles that are then delivered to lysosomes for degradation. Several themes have been emerged from studies of the role of autophagy in cancer to date that will influence future efforts to understand the role of autophagy in tumor biology.

First, autophagy may initially contribute to prevention of oncogenic transformation in pre-malignant cells by eliminating potential sources of oncogenic transformation from the cell. Second, once tumors form and begin proliferating, autophagy allows tumor cells to survive internal cellular stressors elicited by the harsh microenvironment. Third, numerous studies have shown autophagy to be a resistance mechanism in cancer cells treated with conventional DNA damaging chemotherapy, and, more recently, molecularly targeted therapies and anti-angiogenic therapy. Fourth, the use of autophagy inhibition as a therapeutic strategy designed to maximize the therapeutic potential of other anticancer treatments will require further work to define the ideal autophagy mediators to pharmacologically target and define the molecular features that render a tumor cell able to use autophagy as a survival mechanism.

Acknowledgments

Work was supported by funding to MKA from the American Brain Tumor Association, the American Cancer Society, the James S. McDonnell Foundation, the NIH (5K02NS64167-2), and the UCSF Brain Tumor SPORE. A.J. is a Howard Hughes Medical Institute Research Fellow.

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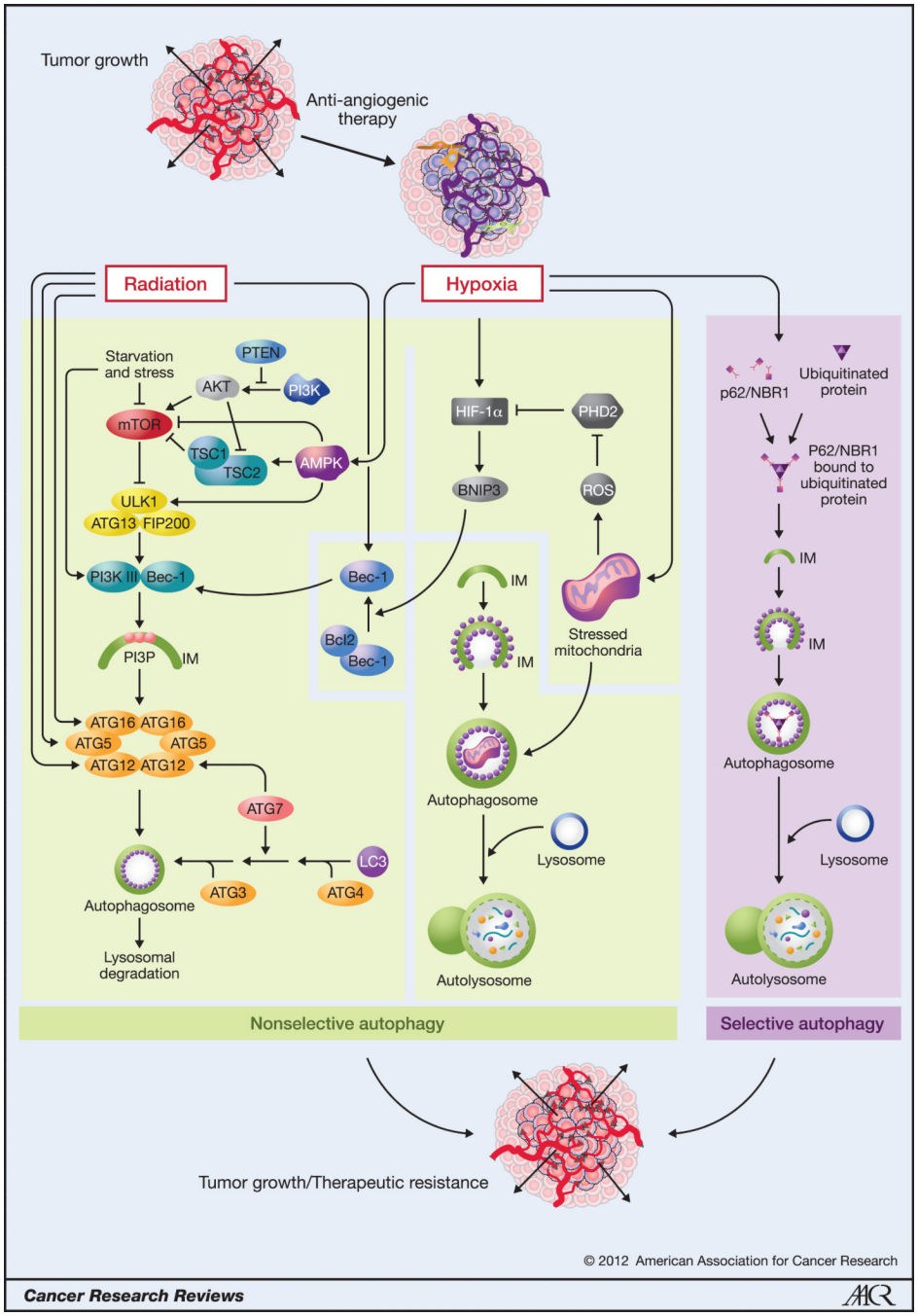

Figure 1. Simplified scheme of nonselective versus selective autophagy, and how they might be impacted in cancer cells by oncogenic pathways and therapy-induced stressors

Shown are regulators of nonselective versus selective autophagy in tumor cells. Hypoxia, as occurs naturally in the tumor microenvironment or as is stimulated by anti-angiogenic therapy, upregulates both nonselective and selective autophagy, with mechanisms more clearly identified for the former. Radiation, another anticancer therapy, has been shown to upregulate factors mediating nonselective autophagy. Abbreviations used: ROS = reactive oxygen species; Bec-1 = Beclin-1; HIF-1α = hypoxia-inducible factor-1α; AMPK = AMP-activated protein kinase; and PHD2 = prolyl hydroxylase domain-containing protein 2.