Tumor Cell Autophagy as an Adaptive Response Mediating Resistance to Treatments Such as Antiangiogenic 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 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 such as chemotherapy and 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 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 that are currently hindered by tumor cell autophagy as a resistance mechanism.

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 although 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 4 steps: (i) formation of the phagophore (also called the isolation membrane) from the endoplasmic reticulum; (ii) assembly of autophagy-mediating proteins at the phagophore; (iii) engulfment of the phagophore by the endoplasmic reticulum to form double-membrane autophagosomes; and (iv) formation of mature degradative vacuoles (autolysosomes) by fusion of autophagosomes with lysosomes. These 4 steps and their mediators can be described as follows:

1. Phagophore formation starts with ATG1, ATG13, and ATG17 forming a complex that recruits membrane protein ATG9 to the developing phagophore. Phagophore formation is aided by the class III phosphatidylinositol-3 kinase (PI-3KIII) Vps34 (vesicular protein sorting 34) and its binding partner, Beclin-1 (ATG6), whereas 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 phagophore 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 phagophore to form the autophagosome.

3. In autophagosome formation, (i) ATG4-mediated cleavage of cytosolic microtubule-associated protein light chain 3 (LC3; mammalian homolog of ATG8) generates LC3-I; (ii) LC3-I is activated by ATG7; (iii) activated LC3-I is transferred to ATG3, which conjugates LC3-I with phosphatidylethanolamine to generate LC3-II; and (iv) LC3-II is incorporated into the phagophore membrane, where it promotes fusion of the phagophore 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 the adaptor molecules that bind LC3-II is p62/SQSTM1, which binds to ubiquitinated proteins and promotes the turnover of p62-ubiquitinated protein aggregates.

4. Autophagosomes form mature autolysosomes by fusing with lysosomes. This aspect of autophagy is less well studied, but it requires G protein Rab7 in its GTP-bound state.
Although autophagosomes can sequester cytosolic material, including organelles, nonspecifically in a process called nonselective macroautophagy, there is evidence that a process of selective autophagy can occur as well. During selective autophagy, autophagic degradation of specific protein aggregates occurs (Fig. 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 it converts 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 that lacks features of apoptosis and instead is 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, some investigators have hypothesized that once this barrier has been overcome and a tumor has formed, the tumor will use autophagy as a survival mechanism to overcome the stresses imposed during cancer progression, as well as 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 such as anticancer treatments was suggested in studies in which autophagy inhibitors, such as hydroxychloroquine or 3-methyladenine (3-MA), were shown to sensitize cancer cells to tamoxifen treatment (7), radiation (8), the DNA alkylating agents cyclophosphamide (7) and cisplatin (9), and the tyrosine kinase receptor inhibitor imatinib (10). However, some studies have suggested the converse—that autophagy can be associated with cell death in tumor cells treated with chemotherapy. For example, one report showed that glioblastoma cells treated with dasatinib and temozolomide exhibited increased autophagy and increased cell death, although a definitive correlation between the 2 phenomena was not offered (11). Another report in which glioblastoma cells treated with the pan-Bcl-2 inhibitor (-)-gossypol exhibited increased autophagy, and cell death carried this observation one step further by showing increased cell survival after (-)-gossypol treatment of cells transduced with short hairpin RNA (shRNA) targeting beclin1 or ATG5 (12). In a study of 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 the release of activating transcription factor (ATP) from dying cells (13).

It is possible that the differences among studies associating autophagy with tumor cell survival versus tumor cell death in response to therapies may merely reflect the extent of the damage induced. Damage below the threshold of tolerance may allow autophagy to be associated with tumor cell survival, whereas damage beyond the threshold of tolerance may cause autophagy to promote tumor cell death. Ultimately, further work will be needed to clarify the mechanisms that render autophagy protective rather than cytotoxic, as well as the molecular basis of the variable role played by autophagy in different cell types (14, 15).

The variable role of autophagy in different tumors could reflect the fact that some tumor cells exhibit loss of autophagy function through mechanisms that remain to be identified but likely include interactions between tumor-suppressor/promoter genes and autophagy, as described below:

1. The mTOR pathway negatively regulates autophagy. A major (and perhaps the primary) regulator of autophagy is the mTOR pathway. mTOR is activated downstream of phosphoinositide 3-kinase (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 that are deficient in PTEN, a tumor suppressor that inhibits mTOR by way of PI3–AKT inhibition, exhibit suppressed formation and maturation of autophagosomes (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. The epidermal growth factor receptor (EGFR)–Ras–mitogen-activated kinase (MAPK) pathway promotes autophagy. Murine embryonic fibroblasts 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 EGFR–Ras–MAPK signaling pathway is altered in several tumors, including >40% of glioblastomas (21).

3. p53 mutations promote autophagy when they are localized to the cytoplasm. p53 mutant proteins that localized to the cytoplasm of colon cancer cells were shown to promote autophagy (22). Mutations in the DNA-binding regions of p53 did not affect autophagy, suggesting that unidentified molecular features of p53 account for its autophagy suppression.

In cancer, hypoxia is a crucial cellular stressor faced by proliferating cells in a microenvironment that contains abnormal vessels that fail to deliver a blood supply effectively to the tumor. Recent reports suggest that the cellular stress of...
hypoxia activates autophagy. Pathways that are activated by the tumor cell response to hypoxia and that have been shown to contribute to autophagy include those mediated by HIF-1α, which is activated during physiologic hypoxia (0.1%–3% O2), or by HIF-1α-independent 5' AMP-activated protein kinase (AMPK), which is activated during anoxia (≤0.01% O2). AMPK acts as a ubiquitous sensor of cellular energy status by responding to an ATP-depleted adenine nucleotide pool by

Figure 1. Simplified scheme of nonselective versus selective autophagy, and how they might be affected in cancer cells by oncogenic pathways and therapy-induced stressors. Shown are regulators of nonselective and selective autophagy in tumor cells. Hypoxia, as occurs naturally in the tumor microenvironment or as stimulated by antiangiogenic 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. Bec-1, Beclin-1; PHD2, prolyl hydroxylase domain-containing protein 2; ROS, reactive oxygen species.
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 (Fig. 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, because 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. Activation of the AMPK pathway increases tumor cell autophagy indirectly through inactivation of the mTOR complex (27) and directly through stimulation of ULK1 (Fig. 1; ref. 28). The ability of tumor cells to use these 2 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 the 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 the 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, such as histone deacetylase inhibitors (31) and cisplatin (32), induce autophagy by increasing the production of ROS in mitochondria.

These observations, which point to autophagy as an adaptive response to radiation therapy and conventional DNA-damaging chemotherapy, are augmented by our recent finding that autophagy is an adaptive response to a different type of therapy, antiangiogenic 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 the VEGF neutralizing antibody bevacizumab in glioblastoma multiforme (GBM), 40% to 60% of tumors progressed after initially successful treatment (35), consistent with the development of resistance to antiangiogenic therapy, a state that involves a poor prognosis and poor response to available treatments (36). We found that hypoxia increases after the devascularization caused by antiangiogenic therapy, consistent with the goals of these therapies, but some tumor cells survive the hypoxic insult elicited by antiangiogenic 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 antiangiogenic therapy can be expanded to determine the effect of hypoxia on cells in the tumor microenvironment. For example, we have found that 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 antiangiogenic therapy was suppressed (33), and suggesting that tumors grow during antiangiogenic therapy without increased endothelial survival. Furthermore, because hypoxia increases the size of the cancer stem cell population (37), one could hypothesize that hypoxia promotes autophagy in cancer stem cells. Confirmation of this hypothesis would provide an additional rationale for inhibiting autophagy to prevent resistance to antiangiogenic treatment.

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

**Autophagy Inhibition in Cancer**

Based on the preclinical evidence mentioned above, autophagy inhibition is currently being investigated as a means of modulating the response to cancer therapies in patients. Currently, the only U.S. Food and Drug Administration–approved agents that are able to inhibit autophagy are chloroquine, an antimalaria drug, and its derivative hydroxychloroquine, both of which block autophagy by disrupting lysosome acidification. One notable completed study was a randomized trial that combined chloroquine with conventional treatment, defined as radiation plus temozolomide, for glioblastoma. The median survival was 24 months with chloroquine treatment and 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 the sample size (30 patients total) was too small to achieve statistical significance, or it could mean that the observed difference was due to chance and there really was no effect from combining chloroquine-mediated autophagy inhibition with standard glioblastoma treatment. If the latter is true, it could be because chloroquine failed to sufficiently inhibit autophagy in patients or because the role of autophagy in the temozolomide response may not be cytoprotective, as suggested by preclinical evidence (29).

Currently, 22 phase I/II cancer clinical trials involving chloroquine or hydroxychloroquine are open nationwide (www.clinicaltrials.gov), including 2 combining hydroxychloroquine with bevacizumab and conventional DNA-damaging therapy.
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The results of these trials could support our preclinical data showing a role for autophagy in resistance to antiangiogenic therapy.

In addition to these ongoing clinical efforts, the use of autophagy inhibition as a therapeutic strategy in cancer may require further preclinical evaluation to optimize the chances of success. The challenges involved in using autophagy inhibition as a therapeutic strategy include (i) recognizing the dual roles played by autophagy in tumors [i.e., cytoprotective or cytotoxic, depending on the tumor type and whether the tumor is in early or late stages of oncogenesis (41)]; and (ii) recognizing the functional autophagy status in tumors, because some tumors may have defects in the autophagy pathway (13) and others may have a preserved capacity for autophagy. The first challenge leads most observers to suggest that autophagy inhibition will be ineffective as monotherapy because basal autophagy may be cytotoxic, whereas stress-induced autophagy (as seen in response to traditional chemotherapy) may be cytoprotective, a response resistance 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 to enable the identification of tumors that are 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 believed that autophagy inhibition will likely be of minimal utility as monotherapy. Therefore, the clinical trials of chloroquine and hydroxychloroquine to date have all combined these agents with treatments that induce autophagy as an adaptive responsive.

Additional preclinical work will also be needed to develop autophagy inhibitors beyond chloroquine or hydroxychloroquine. Although preclinical studies, including ours, have suggested that these agents disrupt autophagy in animal models, other studies have shown that chloroquine may potentiate the effects of autophagy-inducing chemotherapies independently of autophagy disruption (42). Furthermore, it remains to be shown that chloroquine or hydroxychloroquine can effectively block autophagy in human tumors, and how the genetic makeup of these tumors influences their susceptibility to these agents. Should chloroquine or hydroxychloroquine ultimately prove to be too nonspecific for clinical use as autophagy inhibitors, the development of more specific autophagy inhibitors will require a focus on kinases (e.g., ATG1 or Vps34) or proteases (e.g., ATG4) that can specifically regulate the activation of autophagy and autophagosome formation and that have minimal intracellular roles outside of autophagy.

Conclusions

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 emerged from studies of the role of autophagy in cancer that will influence future efforts to understand the role of autophagy in tumor biology.

First, autophagy may initially contribute to the prevention of oncogenic transformation in premalignant cells by eliminating potential sources of oncogenic transformation from the cell. Second, once tumors form and begin to proliferate, autophagy allows the 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 antiangiogenic 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 enable a tumor cell to use autophagy as a survival mechanism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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