

The Four Faces of Autophagy: Implications for Cancer Therapy

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Abstract

It is generally thought that autophagy has two primary and opposing functions in tumor cells in response to stress induced by chemotherapy or radiation. One is the cytoprotective function that can in theory be inhibited for therapeutic advantage by sensitizing the cells to these treatment modalities. The other is the cytotoxic function that is generally not observed with conventional treatment modalities, but that may function to promote tumor cell killing either alone or in association with apoptosis. In this commentary/review, we advance the premise that autophagy is actually populated by at least two additional players. One we have termed the nonprotective form of autophagy, where the cell is apparently carrying out autophagy-mediated degradative functions, but where autophagy inhibition does not lead to perceptible alterations in drug or radiation sensitivity. The other is what we now term the cytostatic form of autophagy in that its activation results in prolonged growth inhibition as well as reduced clonogenic survival (loss of reproductive capacity) but in the absence of actual loss of cell viability through apoptosis or necrosis; however, as is the case with cytotoxic autophagy, inhibition of cytostatic autophagy protects the tumor cell from the agent (drugs or radiation) that promotes the autophagic response. In view of current clinical efforts to exploit autophagy inhibition as a therapeutic strategy for sensitization of malignancies to chemotherapy and radiation, it is critical to recognize that if chemotherapy and/or radiation actually promote autophagy in patient tumors, the autophagy is not of necessity cytoprotective in function. *Cancer Res*; 74(3); 647–51. ©2014 AACR.

Introduction

It is perhaps best to begin by indicating that this article is not designed to provide a detailed description of the morphologic, biochemical, or molecular aspects of autophagy as there are multiple learned reviews on this topic in the literature. Rather, the goal is to address the relatively controversial question of the function of autophagy in tumors subject to radiation or chemotherapeutic drugs and whether this function or functions can be manipulated in the clinic for therapeutic advantage. The fundamental premise driving this undertaking is that there are in fact, at least 4 functional forms of autophagy and therefore the current focus on the cytoprotective form of autophagy alone may, in some if not most cases, be misleading in terms of the clinical efforts to sensitize malignancies to chemotherapy and radiotherapy through the strategy of autophagy inhibition.

More than 30 years ago, when the multidrug resistance phenotype was first recognized as a tumor cell mechanism that broadly limited the impact/effectiveness of chemothera-

peutic drugs, it was anticipated by many laboratories that this phenomenon could be exploited to sensitize the "drug-resistant" tumor cell to chemotherapy by interfering with drug efflux. This seemed to be a logical and attractive approach, in part because the membrane pump (or in fact pumps as we now recognize) were quite promiscuous in terms of potential substrates; consequently, compounds as diverse as calcium channel-blocking agents could be used to saturate the pump and thereby allow the chemotherapeutic agents to accumulate to therapeutic levels within the tumor cell. Unfortunately, despite decades of effort, this approach has not yet achieved fruition in the clinic despite showing promise in preclinical studies. It was also thought that the pump might represent a unique tumor target; however, it was soon recognized that this pump or its variants also serve critical biologic functions in normal tissues and therefore the ever-present challenge of selectivity in cancer therapy could also hamper any efforts to exploit this type of tumor targeting.

In a sense, the identification of autophagy as a potentially protective response of tumor cells to both chemotherapy and radiation has generated a similar burst of enthusiasm and optimism; specifically, it has been anticipated that interfering with what seems to be a virtually uniform response to stressful challenges such as chemotherapeutic drugs and radiation in tumor cells could provide a new era in the modulation and enhancement of antitumor drug and radiation effectiveness (1, 2). Multiple clinical trials are currently in various stages of progress (including one we have initiated in our own institution) combining chloroquine or hydroxychloroquine (thus far,

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doi: 10.1158/0008-5472.CAN-13-2966

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the only U.S. Food and Drug Administration approved drugs that are being used deliberately to suppress autophagy) with various conventional treatment modalities in efforts to enhance the response to treatment. Although a positive outcome in these trials could potentially represent a breakthrough in cancer therapeutics, we would argue that the combination treatment approach is likely to be somewhat premature in part because of the fact that it is based on insufficient preclinical evidence in animal models (3). Furthermore, there are multiple caveats that may not have been sufficiently considered before the initiation of these trials. These include: (i) our inability to predict whether drugs or radiation are actually generating the cytoprotective form of autophagy in the tumors of the patients undergoing treatment; as indicated below, there are (at least) four functionally different forms of autophagy that could be induced by chemotherapy or radiation-induced stress in the tumor cells, only one of which might serve as a therapeutic target; (ii) it is feasible that autophagy is not actually a common or consistent response to chemotherapy or radiation in patients' tumors; (iii) as an ancillary point to (i) and (ii), we currently have no universally accepted and reliable clinical marker for detecting autophagy induction in patients' tumors and certainly no way to distinguish between the different forms of autophagy; (iv) given their known pharmacokinetics, it is highly uncertain as to whether chloroquine or hydroxychloroquine can actually achieve concentrations in the tumor cells that will effectively inhibit autophagy; in fact, we are inclined to argue that the reason that chloroquine and hydroxychloroquine have proven to be relatively nontoxic to patients is because of the fact that they do not actually suppress autophagy at concentrations that are achieved in normal clinical regimens; and (v) because it has been shown that deficiencies in autophagy are associated with human disease (4), any drug that actually is found to suppress autophagy in tumor cells is likely to be quite detrimental to normal tissue homeostasis because autophagy is clearly necessary for the routine elimination of damaged or dysfunctional proteins. In this context, the issue of drug selectivity again rears its quite ugly head because it is likely that chemotherapeutic drugs and radiation that promote cytoprotective autophagy in tumor cells would do the same in normal tissue (a question that is currently under investigation in our laboratory); consequently, if an agent is actually effective in achieving levels in the circulation that inhibit autophagy in the tumor cells, such an agent would be expected to do the same in normal cells, with the undesirable outcome of an increase in host toxicity. However, it is recognized that as empirical strategies developed in the clinic have permitted the effective utilization of "cytotoxic" chemotherapeutic agents at tolerable levels of toxicity, this could, in theory, eventually prove to be the case for autophagy inhibition.

Focusing on the first point raised above, we will discuss the four different functional forms of autophagy for which we provide an abbreviated summary of salient characteristics in Table 1.

Cytoprotective Autophagy

There is extensive and relatively unequivocal evidence in the literature, including studies from our own laboratory, that both

Table 1. Characteristics of the four functional forms of autophagy

Forms of autophagy	Characteristics
Cytoprotective	<ul style="list-style-type: none"> a. May confer resistance to therapy b. Increased sensitivity to therapy when blocked c. Increased apoptosis when blocked d. Possibly involved in normal tissue homeostasis
Cytotoxic	<ul style="list-style-type: none"> a. Promotes cell death when induced b. Cell death may be associated with subsequent apoptosis c. Reduced sensitivity to therapy when blocked d. Unlikely to mediate actions of conventional therapeutic modalities
Cytostatic	<ul style="list-style-type: none"> a. Mediates growth inhibition b. Results in reduced clonogenic survival c. Potentially associated with senescence d. Involved in tumor growth delay/dormancy?
Nonprotective	<ul style="list-style-type: none"> a. Does not differ in intensity from other forms b. Inhibition does not influence sensitivity to therapy c. Relevance related to efforts to enhance response to therapy through autophagy inhibition

cancer chemotherapeutic drugs and radiation can promote a cytoprotective form of autophagy in tumor cells (5–8). As with the other forms of autophagy, the determination of a cytoprotective function is essentially empirical in that either pharmacologic inhibitors of autophagy (such as chloroquine, bafilomycin, 3-methyl adenine, or ammonium chloride) or genetic silencing or knockdown of autophagy-associated genes (such as Beclin, Atg 5, 7, or 12) increases tumor cell sensitivity to the autophagy-inducing stimulus, usually via the promotion of apoptosis. However, a caveat to these approaches is that although inhibition of autophagy does frequently result in the promotion of apoptosis, this of itself is not sufficient evidence that the autophagy is cytoprotective unless it can be shown that (drug or radiation) sensitivity has actually been enhanced when autophagy has been blocked (e.g., by the performance of clonogenic survival assays). That is, it is possible that the original growth arrest or cell death response to the therapeutic agent that was actually mediated through autophagy is now converted to a response mediated through apoptosis without any actual alteration in sensitivity to treatment. In this context, it is feasible that a cytostatic form of autophagy similar to what is described later could have been overlooked in previous work that has identified cytoprotective forms of autophagy.

Recognition of the existence of this cytoprotective form of autophagy has been the foundation for multiple clinical trials

(as cited in ref. 9) based on the premise that induction of autophagy confers resistance (or at least a reduced degree of sensitivity) to the inducing agent and that consequently interference with or suppression of this autophagy will provide a pathway toward an enhanced response to treatment. However, returning to the issue of selectivity that was mentioned previously, if an agent is being administered systemically to inhibit autophagy in tumor cells, it must be assumed that autophagy will also be suppressed in normal cells. Given that a number of neurodegenerative diseases are characterized by defective autophagy (4), it is reasonable to assume that autophagy inhibition could prove to be quite detrimental to normal tissue. Even if inhibition of autophagy alone proves not to be detrimental to patients, chemotherapeutic drugs that promote a protective form of autophagy in tumor cells are likely to do the same in normal cells; consequently, it would be expected that autophagy inhibitors would have the capacity to collaterally increase drug toxicity to sensitive normal tissue such as bone marrow. (This would presumably be less of an issue with radiation therapy, which can generally be highly targeted to the tumor.)

The issue of increased toxicity to normal tissue may not prove to be a problem in those clinical situations where autophagy inhibition will be relatively brief and normal cells can recover, perhaps more rapidly than tumor cells (which is considered to be the foundation for the success of many cancer therapeutic strategies). However, this is likely to be a critical issue where the therapeutic design involves prolonged treatment with a drug such as Gleevec coupled with sustained inhibition of autophagy. One logical outcome of this argument is that chloroquine and hydroxychloroquine are unlikely to be appropriate drugs for this purpose because the fact that patients with malaria are able to endure treatment with these drugs for years suggests that chloroquine and hydroxychloroquine may not actually be acting to inhibit autophagy, at least at the doses that are used effectively for malaria treatment.

Nonprotective Autophagy

When recently discussing the possibility of chemotherapeutic drugs and radiation promoting a nonprotective form of autophagy with a long-time collaborator who is quite scientifically rigorous, I was met with no small degree of skepticism about its potential importance or rather the likely insignificance of the nonprotective form. Before further discussing this form of autophagy, I would argue that if preclinical findings relating to cytoprotective autophagy are to be translated to the clinic by developing an effective pharmacologic approach for autophagy suppression in patients, it would seem that we are first obligated to unequivocally determine that the conventional treatment protocol is, in fact, promoting the cytoprotective form of autophagy (an outcome that is likely to be both tumor and drugs specific) before complementing conventional treatment with an autophagy inhibitors. This is analogous to determining whether patients with breast cancer are estrogen receptor positive before treating with tamoxifen or overexpress the HER2 receptor before treating with trastuzumab.

In a previous paper (3), we presented data in breast tumor cells in cell culture indicating that ionizing radiation could promote autophagy, whose inhibition did not alter sensitivity to radiation; we further demonstrated that chloroquine did not sensitize (4T1) murine breast tumor cells to radiation in an immunocompetent animal model. Although we were unable to determine whether radiation promoted autophagy or the chloroquine actually effectively inhibited autophagy in the tumor-bearing animals, it is possible that the lack of sensitization could be related, in part, to Kroemer's findings that autophagy inhibition interferes with the immune system's capability to recognize the tumor undergoing a stress response (10, 11). We also previously cited a number of other reports in tumor-bearing animals where it was relatively clear that the strategy of autophagy inhibition was ineffective (3). These observations indicate that, other concerns aside, it is imperative that it be determined that a particular treatment or combination of treatments is generating a cytoprotective form of autophagy in the patient's malignancy before efforts to suppress autophagy are considered. In this context, it is possible that cytoprotective autophagy could occur preferentially in tumors that are autophagy addicted through, for example, overexpression of ras as in pancreatic cancers (12, 13). Nevertheless, given the fact that there is no uniformly accepted methodology for assessing autophagy in clinical samples, let alone defining the form of autophagy should it be occurring, the outcomes of the current clinical trial efforts are likely to be difficult to interpret in relation to the underlying concept of inhibition of cytoprotective autophagy.

Cytotoxic Autophagy

In recent studies from our own laboratory, we have reported that vitamin D (or the vitamin D analog, EB 1089) can be combined with radiation to promote a cytotoxic form of autophagy in breast tumor cells (7, 8). As discussed in a recent review, a number of other laboratories have also reported on the generation of cytotoxic autophagy that either kills cells of its own or acts as a precursor to apoptosis (2). In this context, it is critical to note that the different forms of autophagy are currently distinguished based primarily, if not exclusively, on their functional characteristics while being essentially indistinguishable based on morphologic, biochemical, or molecular profiles that might be used to identify one or another functional form. Functionally, cytotoxic autophagy is associated with a reduction in the number of viable cells and/or reduced clonogenic survival upon treatment. Fundamentally, however, the difference between cytotoxic and cytoprotective autophagy is that when cytoprotective autophagy is inhibited, the cells are sensitized to the treatment modality; conversely, when cytotoxic autophagy is inhibited, the cells become less sensitive to the treatment modality. It would seem intuitive that when autophagy exhibits a cytotoxic function, this would be related to the autophagy being particularly extensive and prolonged, as the self-cannibalism that is a hallmark of autophagy cannot, by definition, be sustained. However, there is as yet no data that would support this contention and we might speculate that differences in autophagic function will be found to relate to

specific signaling pathways and/or substrates for the autophagic machinery (a premise currently under active investigation in our laboratory).

In a seminal paper by the Kroemer laboratory, the putative cytotoxic actions of autophagy for conventional antitumor drugs were largely laid to rest (14). These studies demonstrated that blocking autophagy induced by a host of therapeutic agents by knockdown of ATG7 did not result in protection from their antiproliferative or cytotoxic actions. This finding, if it can be extrapolated to the clinical situation, would support the potential utility of autophagy inhibition as a therapeutic strategy if, in fact, conventional drugs (and possibly the hypoxic tumor environment) promote solely the protective form of autophagy. This strategy of blocking autophagy further assumes that autophagy is actually a consequence of therapy in human malignancies, which has not, to our knowledge, been proven. However, as also shown by Kroemer's group in animal studies (10, 11), tumor cells undergoing autophagy secrete factors that activate an immune response that is critical for drug effectiveness. Consequently, a pharmacologic approach that is actually effective in suppressing autophagy would be at best counterproductive and might actually interfere with the utility of conventional treatments by attenuating the immune response.

Cytostatic Autophagy

Recent studies in our laboratory designed to extend our findings relating to radiation sensitization by vitamin D in breast tumor cells to non-small cell lung cancer cells have identified an additional form of autophagy that we have termed "cytostatic" (manuscript in preparation). As with the other forms we have discussed, the identification of this form of autophagy is entirely functional and empirical. The combination treatment of vitamin D (or the vitamin D analog, EB 1089) with radiation results in a more pronounced growth inhibition of non-small cell lung cancer cells than for radiation alone, suppresses the proliferative recovery that occurs with radiation alone, and markedly shifts the clonogenic survival curve for radiation, indicative of increased sensitivity to radiation. Similar to the impact on cytotoxic autophagy in breast tumor cells, pharmacologic inhibition of autophagy with either chloroquine or 3-MA protects the cells from the sensitization to radiation by vitamin D or EB 1089. What distinguishes this form of autophagy from the cytoprotective function is that we fail to detect evidence of cell killing that we reported in the breast tumor cells. [The relationship of this form of autophagy to the well-characterized and prolonged growth arrest associated with senescence (15) is currently under investigation in our laboratory.]

It should not be surprising or unexpected to identify a cytostatic form of autophagy because the historical function of autophagy occurring under conditions of nutrient deprivation has been to permit cellular survival in a state of stasis where the metabolic state is maintained (16). Although the possibility that cytoprotective autophagy also has a cytostatic component should be considered, investigators have generally not determined whether cells undergoing cytoprotective

autophagy in response to chemotherapy or radiation are actually growth arrested. Nevertheless, it must be emphasized that the form of cytostatic autophagy we have recently identified is quite different from the cytoprotective form. In the same (A549 and H460) cells where we as well as Kroemer's group can demonstrate cytoprotective autophagy by radiation alone (11), the addition of vitamin D or EB 1089 converts cytoprotective autophagy to cytostatic autophagy. It is furthermore characterized by loss of the cytoprotective function of radiation alone, which might otherwise complicate efforts to inhibit the cytostatic form; that is, reversal of the sensitization induced by vitamin D or EB 1089 by autophagy inhibition would be masked by collateral sensitization through interference with the cytoprotective form (7, 8).

The therapeutic implications of cytostatic autophagy would seem to be similar to those for the cytotoxic form. That is, if chemotherapeutic agents or radiation are promoting prolonged and sustained growth arrest in the tumor cells (and possibly generating danger signals that activate an immune response), then suppression of autophagy is likely to reduce the impact and effectiveness of the therapy.

Summary and Conclusions

It is proposed that there are (at least) 4 functional forms of autophagy that may occur in response to chemotherapy or radiation, cytoprotective, cytostatic, cytotoxic, and nonprotective. These forms are likely to be context (tumor cell line and agent) specific and it is currently not possible to predict which form will be induced by a particular therapy either in preclinical studies and certainly not in the clinic. In part this is because of the fact that at least currently, these forms of autophagy currently have no clear-cut morphologic, biochemical, or molecular distinctions. An additional issue that is likely to hinder making clear-cut interpretations of the clinical data is that there is no currently accepted protocol for evaluating whether (any form of) autophagy is occurring in a patient's tumor either based on a biopsy (recognizing the challenges this would entail) or preferably based on biomarkers in the circulation. Assuming the observations of differing forms of autophagy in experimental tumor lines are relevant to the clinic (and admittedly there is no current evidence whether this is, in fact, the case), the concept that autophagy inhibition could sensitize malignancies to therapy would only occur only in those cases where the therapy-induced autophagy has a cytoprotective function, which could in theory be, for example, limited to tumors that are autophagy addicted. An additional concern relating to the use of chloroquine or hydroxychloroquine in clinical trials for the inhibition of autophagy is whether the pharmacokinetics of tolerable doses of these drugs will be sufficient to promote autophagy in the tumors. Furthermore, if another agent or agents are identified that are proven effective in suppressing autophagy in the tumor cell, there is little certainty that concomitant interference with autophagy in normal cells might not disturb their homeostasis to the point where toxicity is a serious concern. That is, it is possible that the antimalarial drugs chloroquine and hydroxychloroquine have been utilized with minimal toxicity for many decades

precisely because these agents are not actually capable of interfering with autophagy at the doses that are generally considered tolerable. Finally, there has been little if any effort to discern whether chemotherapy and/or radiation promote autophagy (presumably the cytoprotective form) in normal cells. If this is, in fact, the case, there would seem to be little likelihood that blocking autophagy could be tumor selective.

In summary, it may be that current clinical trials are based on insufficient information about the forms of autophagy that could be induced in the clinic by conventional therapy; furthermore, even in the event that autophagy is actually cytoprotective in some cases, hydroxychloroquine may not be capable of achieving sufficient intratumoral levels to suppress autophagy and enhance sensitivity to treatment. Finally, if the studies in animal models indicating a requirement for a functional immune response to an autophagy signal can be translated to the clinic, autophagy inhibition could prove to be counterproductive. If the current clinical trials prove disappointing because of inadequate design, an unfortunate outcome might be that rigorous and meticulous studies to determine whether autophagy inhibition could actually prove to be a viable therapeutic strategy might be stymied or abandoned through misinterpretation of the reasons that the trials have failed. Conversely, if a few trials prove to be successful, their success may actually have a different explanation than the conclusion that cytoprotective autophagy has been suppressed. In those cases where the addition of chloroquine or

hydroxychloroquine to conventional therapy might more effectively suppress tumor growth than conventional drug or radiation treatments alone, we do not have sufficiently sensitive technology to surmise whether this outcome has occurred through autophagy inhibition or another type of (off-target?) interaction between the hydroxychloroquine and the established therapy. In such case, additional efforts to suppress autophagy would likely be initiated utilizing hydroxychloroquine, which may be inappropriate for this purpose. Consequently, the outcome of the current trials, whether success or failure, must be coupled with efforts to analyze and discern whether autophagy has been induced by therapy alone, whether autophagy has actually been inhibited by the coadministration of chloroquine/hydroxychloroquine, and whether the sensitization that is likely to be observed in at least some clinical trials is actually a consequence of or at least directly related to inhibition of the cytoprotective form of autophagy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This publication was supported by CTSA Award #UL1TR000058 from the National Center for Advancing Translational Science.

Received October 14, 2013; revised November 14, 2013; accepted November 15, 2013; published OnlineFirst January 23, 2014.

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