Cell, Vol. 120, 159–162, January 28, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.01.005

Eating Oneself and Uninvited Guests: Autophagy-Related Pathways in Cellular Defense

Minireview

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The eukaryotic cell uses an evolutionarily conserved lysosomal pathway of self-digestion (autophagy) for survival when extracellular nutrients are limited. In this issue of *Cell*, new evidence indicates that autophagy is used to for survival when intracellular nutrients are limited by growth factor deprivation (Lum et al., 2005). Other recent studies indicate that the autophagy machinery is also used to degrade foreign microbial invaders (xenophagy).

A fundamental event in eukaryotic evolution was the development of internal membranes that compartmentalized the cell's constituents. This cytomembrane system not only directed specific cellular functions to specific regions of the cell but also mediated the transition from the extracellular digestion of food (by prokaryotes) to the intracellular digestion of food (by primitive eukaryotes). The internal digestion of food freed eukaryotic cells from the need to be in continuous contact with their food and allowed them to invade new kinds of habitats, thus facilitating evolutionary diversity.

The capacity for intracellular digestion also created vulnerabilities for the eukaryotic cell. First, by not living in continuous juxtaposition to the food supply, eukaryotes risked periods of nutrient starvation. When they evolved to the metazoan level, individual cells were at the mercy of other cells in the organism both for the generation of extracellular nutrients and for permission (delivered in the form of growth factors) to use such nutrients. Second, since the endolysosomal system could also be exploited by invading hostile microorganisms, the eukaryotic cell needed mechanisms for self-defense against intracellular pathogens.

Intriguingly, these vulnerabilities may have been partly addressed by additional evolutionary events associated with the cytomembrane system. The risk of starvation was addressed by directing the cytomembrane system to digest its own cellular contents, a process known as autophagy (derived from the Greek words meaning to eat oneself). The risk of pathogen invasion was addressed by directing the cell's digestive machinery to the breakdown of invading microorganisms, a process herein referred to as xenophagy (derived from the Greek words meaning to eat foreign matter).

In this issue of *Cell* and in other recent studies, scientists report new evidence that these ancient pathways play essential roles in protecting mammalian cells against different forms of nutrient stress and infection with different human bacterial pathogens.

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Neonatal Starvation and Growth Factor Deprivation: Autophagy to the Rescue

The concept that autophagy sustains survival during nutrient stress is not new. Almost five decades ago, autophagosomes were found to be more common in the livers of starved than in the livers of well-fed animals (reviewed in deDuve and Wattiaux [1966]). This finding led to the notion that autophagy, through the bulk lysosomal degradation of cytoplasmic proteins and organelles (see Figure 1), is the major catabolic pathway that eukaryotic cells use to generate intracellular nutrients to maintain energy production and macromolecular synthesis when external nutrients are limited. One of the yeast genetic screens that identified the evolutionarily conserved autophagy (*ATG*) genes isolated mutants that died during nitrogen or carbon deprivation. Autophagy genes in higher eukaryotes are also necessary for survival during starvation in *Dictyostelium*, for survival during dauer diapause in *C. elegans*, and for preventing starvation-induced chlorosis in plants (reviewed in Levine and Klionsky [2004]).

It is easy to imagine how organisms that reside in soil (e.g., slime molds, nematodes, and plants) may routinely encounter periods of starvation when the recycling of cellular constituents via autophagy is required for survival. However, it has been less clear if mammals are inevitably exposed to periods of extreme starvation when autophagy is essential. The neonatal period, when the trans-placental nutrient supply is suddenly interrupted and the neonate has not yet had adequate time to replenish nutrients through maternal milk, may be one such critical time in mammalian life.

For decades, it has been believed that autophagy allows the newborn liver to degrade glycogen, an important metabolic substrate during the neonatal period. Recently, Kuma et al.—using mice expressing a transgenic fluorescent autophagy marker, GFP-LC3—found a massive increase in autophagy immediately after birth in tissues that have a sudden increase in energy requirements (e.g., heart and diaphragm) or confront a drastic change in environment as they transition from amniotic fluid to air (e.g., lung alveolar cells and skin) (Kuma et al., 2004). This pattern was distinct from that observed in GFP-LC3 transgenic adult mice following starvation (in which skeletal muscle is the primary site of increased autophagy) (Mizushima et al., 2004) and, surprisingly, did not include the liver, a site previously shown by electron microscopic analyses to be autophagically active during the neonatal period.

Kuma et al. showed that the autophagic machinery was critical for sustaining life during the neonatal period. Neonatal mice that lack Atg5, an acceptor molecule for the ubiquitin-like molecule Atg12, had no autolysomes (degrading autophagic vacuoles) in their tissues and died within the first day of life. Since these mice also had a suckling defect, Kuma et al. evaluated their survival independently of their ability to nurse. Following delivery, they found that wild-type mice survived approximately 20 hr if they were not fed and at least 72 hr if they were artificially fed. In contrast, *atg5*-deficient

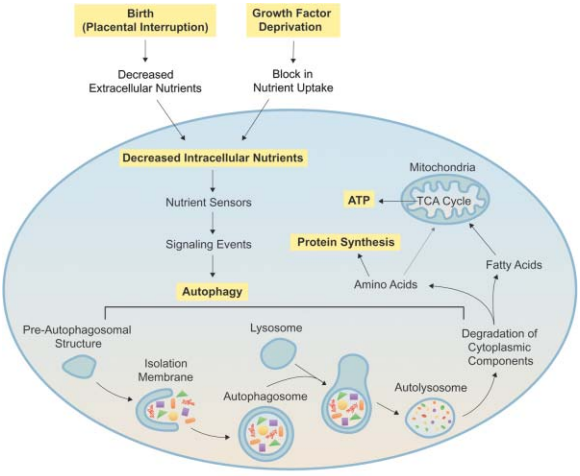

Figure 1. Mammalian Autophagy in Cellular Defense against Two Forms of Nutrient Stress: Birth and Growth Factor Deprivation

neonates survived about 12 hr if they were not fed and about 25 hr if they were artificially fed. Both wild-type and *atg5*$^{-/-}$ neonates were comparably hypolipidemic and hypoglycemic (challenging a role for hepatic glycogen autophagy in maintaining glucose supply). However, after starvation but not at birth, *atg5*$^{-/-}$ neonates had decreased plasma and tissue concentrations of amino acids as well as increased cardiac activation of AMP-activated protein kinase, a nutrient energy sensor of the intracellular AMP:ATP ratio. Based on these observations, Kuma et al. proposed that neonates depend upon the amino acids produced by autophagy for the maintenance of energy homeostasis and survival.

The pattern of autophagy induction during the neonatal period poses some interesting questions. Since local amino acid concentrations in the heart, liver, and brain paralleled systemic concentrations, one wonders why local autophagy is required in tissues such as the heart and diaphragm that have a sudden increase in energy needs at birth. Presumably, increased autophagy at more classical catabolic sites (e.g., skeletal muscle or liver) could provide an adequate systemic supply of nutrients to tissues with increased energy needs. Therefore, the massive induction of autophagy in the heart and diaphragm (which is not observed during short-term starvation in adult mice) may indicate that the increased energy needs of these organs at birth cannot be met solely by extracellular nutrients; rather, local autophagy may be required to generate sufficient intracellular metabolic substrates to sustain cellular energy homeostasis. Autophagy at these and other sites (such as the skin and lungs for which there is no obvious increased energy need) may also reflect a role for autophagy in non-nutritional forms of environmental adaptation that must occur for neonates to survive. Furthermore, given their suckling defects, *atg5*$^{-/-}$ mice may also possess other developmental abnormalities that contribute to their neonatal lethality.

The requirement for autophagy in maintaining cellular energy homeostasis in mammals may not be restricted to settings when extracellular nutrients are limited. In previous work, Thompson and colleagues found that an adequate extracellular nutrient supply does not ensure adequate intracellular energy substrates for mammalian cells (see Lum et al. [2005] for references). Growth factors are essential to license cells to take up extracellular nutrients by increasing the cell surface expression of nutrient transporters. Now, in the present issue of *Cell*, the same group reports that, in the absence of growth factor, autophagy is essential in prolonging mammalian cell survival (Lum et al., 2005).

An increase in autophagosomes has been observed in mammalian tissues following the abrupt withdrawal of hormonal trophic factors, including the prostate following castration, the ovarian corpus luteum during postpartum regression, and the mammary gland during postlactation involution. In fact, the presence of massive amounts of cellular autophagy in these settings has contributed to the classification of autophagy as a form of non-apoptotic programmed cell death. However, it is not known whether autophagy contributes to cell death in such settings or represents a failed effort to preserve cell viability. Since apoptosis rapidly kills cells following trophic factor deprivation, the protective functions of autophagy could easily be masked.

Using cells lacking the core apoptotic machinery derived from *bax*$^{-/-}$, *bak*$^{-/-}$ animals, Lum et al. dissected an independent role of autophagy in maintaining cellular energy homeostasis and survival following withdrawal of the growth factor, IL-3. Whereas wild-type hematopoietic cell lines rapidly die following IL-3 withdrawal, *bax*$^{-/-}$, *bak*$^{-/-}$ cell lines survive for several weeks. In the IL-3-deprived *bax*$^{-/-}$, *bak*$^{-/-}$ cells, the decline in ATP levels was less than expected based upon the magnitude of the decline in the cell surface expression of the major glucose transporter GLUT1. This suggested that the IL-3-deprived *bax*$^{-/-}$, *bak*$^{-/-}$ cells used alternative substrates generated by cellular catabolic pathways to maintain energy production. Indeed, IL-3 deprivation was accompanied by induction of autophagy, a decrease in cell size, and a gradual disappearance of recognizable cytoplasmic structures. Autophagic breakdown of cellular constituents was critical for maintaining cellular energy homeostasis and cell survival since inactivation of the autophagy genes, *atg5* and *atg7*, or pharmacological blockade of autophagy resulted in a rapid decline in ATP and cell death.

Thus, autophagy appears to play critical roles in protecting mammalian cells against deficiencies of extracellular and intracellular nutrients (Figure 1). The specific signaling events regulating the induction of autophagy during neonatal starvation and IL-3 deprivation have not been defined but may involve the evolutionarily conserved nutrient sensors, GCN2 (which stimulates autophagy) and TOR (which inhibits autophagy) (see Codogno and Meijer [2004] for review). Additional work is needed to precisely define how autophagy affects cellular metabolism and bioenergetics to sustain survival during nutrient stress. It will also be important to assess whether the pro-survival function of autophagy during growth factor deprivation is conserved in cells with intact apoptotic machinery. If so, such a finding will pose a major challenge to the decades-old belief that autophagy is a mechanism of cell death following trophic factor withdrawal.

Bacterial Invasion: Xenophagy to the Rescue

The concept that intracellular pathogens may be degraded by an autophagy-like pathway is not novel. It has already been postulated that intracellular bacteria, (e.g., *Rickettsia conorii*, *Listeria monocytogenes*, and *Mesorhizobiuim huakuii*) are degraded by autophagosomes (reviewed in Kirkegaard et al., [2004]). In addition, the autophagy gene, *beclin 1*, has antiviral activity in mammalian cells and plants, and the antiviral PKR signaling pathway stimulates autophagy (reviewed in Seay et al. [2005]). Furthermore, cytokines that play key roles in resistance to intracellular pathogens (e.g., γIFN, and TNF) induce autophagy.

Until recently, however, it has been difficult to prove that intracellular bacteria are degraded by an autophagy-like pathway. Before the discovery of individual components of the autophagic machinery, there were no markers to unequivocally identify autophagosomes. Furthermore, even if bacteria were observed within putative autophagosomes, it was hard to follow the fate of such structures. Moreover, several bacteria (and viruses) hijack components of the autophagic machinery to establish their own protected replicative niches; in such cases, membranous sequestration may foster the growth of the organism rather than promote its degradation through a lysosomal pathway (reviewed in Kirkegaard et al. [2004] and Shintani and Klionsky [2004]). In addition, prior to the identification of the autophagy genes, it was not possible to directly assess the consequences of the lack of autophagy on the life cycle of intracellular pathogens.

Using specific markers for autophagosomes as well as cells lacking autophagy genes, recent studies provide new evidence that the autophagic machinery plays a role in the degradation of both extracellular bacterial pathogens that invade the cell (e.g., Group A *Streptococcus*) (Nakagawa et al., 2004) and true intracellular bacterial pathogens (e.g., *Mycobacterium tuberculosis* and *Shigella flexneri*) (Gutierrez et al., 2004; Ogawa et al., 2004). However, the utilization of the autophagic machinery in these circumstances differs somewhat from classical autophagy. Classical autophagy is generally thought to be nonselective, involving the bulk degradation of cytoplasmic contents. In contrast, during the host response to intracellular bacteria, the autophagic machinery appears to selectively sequester bacteria (or compartments containing bacteria). Additionally, at least in the case of *M. tuberculosis* infection (and potentially other pathogens that replicate inside vacuoles), the autophagic-like degradation process may not involve the sequestration of cytoplasmic contents by an isolation membrane. Therefore, the term xenophagy distinguishes the utilization of the autophagic machinery for the degradation of intracellular pathogens from classical autophagy.

The Group A *Streptococcus* (GAS) is an extracellular pathogen that causes human diseases by its capacity to bind to extracellular matrix proteins and to produce a wide range of toxins. It can invade nonphagocytic cells (e.g., epithelial cells, keratinocytes), but once inside cells, GAS cannot proliferate and is degraded by mechanisms that until recently have been unidentified. The biological significance of GAS invasion is not known, but it has been proposed that cellular entry may protect

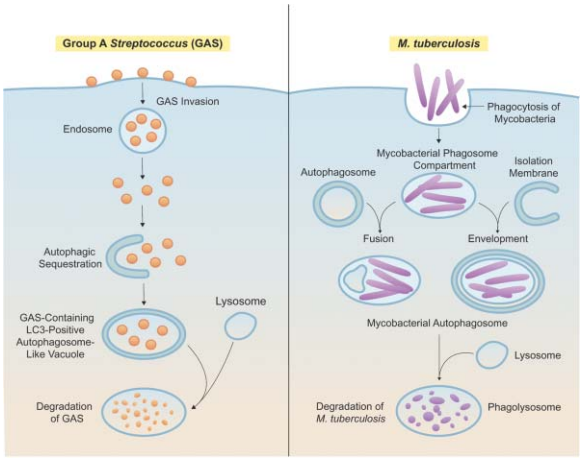

Figure 2. Xenophagy in Cellular Defense against an Invading Extracellular Pathogen, Group A *Streptococcus* (GAS), in a Nonphagocytic Cell and an Intracellular Pathogen, *M. tuberculosis*, in a Phagocytic Cell

GAS from host clearance mechanisms or from antibiotics (reviewed in Bisno et al. [2003]).

Nakagawa et al. show that internalized GAS is targeted for rapid degradation by xenophagy, thus defying the notion that GAS escapes host clearance mechanisms by seeking intracellular refuge (Nakagawa et al., 2004). In wild-type mammalian cells, internalized GAS associates within a few hours with multimembranous vacuolar structures that contain the autophagosomal marker, LC3, and at later time points, with single membrane structures that resemble lysosomes. The LC3-positive vacuoles are morphologically distinct from classic autophagosomes in that they are significantly larger and contain primarily GAS rather than cytoplasmic organelles. In cells lacking *atg5*, these vacuoles are absent and there is a decreased rate of degradation of intracellular GAS.

The authors propose that GAS entry into the cytoplasm triggers sequestration by autophagosome-like compartments (see Figure 2). This model fits best with their finding that a mutant strain of GAS that cannot escape from endosomes does not enter LC3-positive autophagosome-like compartments. However, the possibility that GAS-containing endosomes directly fuse with autophagosome-like compartments cannot be excluded since this mutant strain could be defective in xenophagy induction, independently of its inability to escape from endosomes. This question and other interesting questions regarding the cell biology of the xenophagic degradation of GAS remain. For example, what triggers the induction of xenophagy? What is the source of the membranes for the autophagosome-like compartment? What are the molecular determinants for the selectivity of the process?

From a bacterial pathogenesis perspective, the significance of xenophagic degradation of internalized GAS is unknown. In the absence of autophagy, Nagakawa et al. observed a modest short-term increase in the numbers of intracellular bacteria in *atg5*$^{-/-}$ cells. However, it is not yet clear whether GAS could survive long-term within the cell and persistently replicate in the ab-

sence of xenophagy; the authors only measured bacterial counts for 4 hr following infection, and at the 4 hr time point, bacterial colony counts were significantly reduced, even in *atg5*$^{-/-}$ cells (suggesting the presence of additional, xenophagy-independent mechanisms of GAS degradation). Furthermore, it is not known whether delayed degradation of intracellular GAS in xenophagy-deficient cells would increase bacterial virulence, either by damaging the cells it had invaded or increasing extracellular levels of organisms. Studies in autophagy-deficient organisms will be useful in assessing the role of xenophagy in host defense against GAS and other microorganisms. Perhaps degradation by xenophagy represents a novel mechanism by which nonphagocytic cells help to clear extracellular pathogens.

For true intracellular bacterial pathogens, the role of xenophagy in innate immunity may be more clear. Ogawa et al. have recently demonstrated that the intracellular enteric bacterial pathogen, *Shigella flexneri*, encodes a virulence protein, IscB, that blocks bacterial colocalization with LC3-positive compartments, presumably through its ability to antagonize the interaction of another *Shigella* virulence protein, VirG, with the autophagy protein Atg5 (Ogawa et al., 2004). Mutant bacteria lacking *iscB* demonstrate impaired growth in wild-type cells, but not in *atg5*$^{-/-}$ cells. These findings suggest that xenophagy is involved in anti-*Shigella* innate immunity and that xenophagy can be antagonized by a bacterial virulence factor. Such antagonism is likely to be a common survival strategy of diverse intracellular pathogens.

New evidence indicates that tuberculosis can also be tamed by xenophagy. *M. tuberculosis* infects a professional phagocyte, the macrophage, where it manages to reside long-term in the phagosome by interfering with phagolysosome biogenesis (reviewed in Vergne et al. [2004]). The inhibition of phagosome-lysosome fusion is mediated by mycobacterial lipids that mimic mammalian phosphatidylinositols and inhibit phosphatidylinositol 3-phosphate (PI3P)-dependent membrane trafficking mechanisms. Gutierrez et al. have found that this block in phagolysosomal maturation can be overcome by activating cellular autophagy, either through starvation or inhibition of TOR kinase (Gutierrez et al., 2004). Activation of autophagy reversed the usual acidification defect observed in mycobacterial-containing phagosomes, resulted in colocalization of mycobacterial-containing phagosomes with markers of the late endocytic/lysosomal compartments and with the autophagy proteins (e.g., LC3 and Beclin 1), and decreased mycobacterial survival. It is not yet clear whether the activation of the autophagy pathway results in the fusion of the phagosome with, or envelopment of the phagosome by, an LC3-positive membranous compartment (see Figure 2) or an alternative membrane trafficking pathway.

The stimulation of autophagy as a means of killing *M. tuberculosis* may be an antimicrobial strategy already utilized by the immune system. Confirming previous findings in nonphagocytic cells (Inbal et al., 2002), Gutierrez et al. demonstrated that interferon-γ (IFN-γ), one of the major cytokines associated with protective immunity against *M. tuberculosis*, activates autophagy in uninfected macrophages. Furthermore, IFN-γ treatment increased the percentage of LC3-positive mycobacterial phagosomes in infected macrophages. A critical next step will be to examine whether the antimycobacterial action of IFN-γ (and its action against other intracellular bacteria and viruses) requires autophagy genes. If so, this observation will provide important insights into the mechanisms by which immune mediators promote killing of intracellular pathogens.

Xenophagy may play a role in host defense not only by killing intracellular bacteria but also by enhancing immune recognition of infected cells via the generation of antigenic bacterial peptides. An autophagy-like pathway plays a role in the processing of an endogenously produced viral antigen for presentation by MHC class II molecules to CD4 T lymphocytes (Paludan et al., 2004). It is tempting to speculate that the xenophagic degradation of intracellular bacteria also contributes to antigen presentation by either MHC class I or MHC class II pathways. Interestingly, *M. tuberculosis* inhibits intraphagsomal antigen processing of MHC class II-peptide complexes by mechanisms that overlap with its ability to inhibit phagolysosome biogenesis. An important question is whether overriding this block with autophagy stimulation, in addition to decreasing intracellular mycobacterial survival, also enhances adaptive immunity against *M. tuberculosis*. Such a finding would represent a major advance in our knowledge of how the autophagic machinery orchestrates a coordinated, multifaceted approach to the elimination of intracellular pathogens.

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