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Extracellular Matrix Regulation of Autophagy

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Summary

Integrin-mediated attachment of epithelial cells to extracellular matrix (ECM) is critical for proper growth and survival. Although detachment leads to apoptosis, termed anoikis, recent work demonstrates that ECM detachment also robustly induces autophagy, a tightly regulated lysosomal self-digestion process that actually promotes survival. Autophagy presumably protects epithelial cells from the stresses of ECM detachment, allowing them to survive provided they reattach in a timely manner. Currently, the intracellular signals linking integrin engagement to autophagy remain unclear, but certain growth factor, energy-sensing, and stress response pathways represent attractive candidates. Moreover, autophagy may be a previously unrecognized mechanism utilized by detached cancer cells to survive anoikis, which may facilitate tumor cell dormancy, dissemination, and metastasis.

Introduction

The attachment of epithelial cells to an underlying basement membrane or extracellular matrix (ECM) is vital for cell survival [1]. In normal tissues, the loss of integrin engagement with the surrounding ECM results in an apoptotic death program, termed anoikis [2,3]. However, evidence also indicates that detachment triggers anti-apoptotic signals, which presumably delay the onset of anoikis, allowing cells to survive provided they reestablish ECM contact in a timely manner [4,5]. More recently, studies demonstrate that macroautophagy (hereafter called autophagy), a tightly regulated self-digestion process known to promote cell survival, is robustly induced upon ECM deprivation. This review highlights recent studies demonstrating ECM regulation of autophagy, overviews candidate pathways that may relay signals from disengaged integrins to the autophagic machinery, and speculates on the role of autophagy during dormancy in tumor cells deprived of ECM contact, which may contribute to cancer cell survival, dissemination and metastasis.

Autophagy and Cell Fate

Proper turnover of cytoplasmic proteins and organelles is vital for maintaining cellular homeostasis. Degradation of short-lived proteins is handled primarily by the proteasome while autophagy degrades long-lived proteins and organelles. Although morphologists have observed autophagy for decades, the recent discovery of AuTophagy-related genes (ATGs) has provided the means to manipulate and monitor the autophagic process in experimental models leading to a rapid expansion of knowledge on the functions of autophagy in both normal

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physiology and disease [6]. Autophagy proceeds through multiple morphological stages [7, 8]. First, a double membrane structure, termed an isolation membrane, is formed. This isolation membrane expands to encompass cytoplasmic contents and fuses to form a double membrane vesicle, called an autophagosome. The formation and expansion of the early autophagosomes requires two ubiquitin-like conjugation systems highly conserved from yeast to humans [9] (Figure 1). Finally, the autophagosome and its contents fuse with the lysosome leading to the degradation and recycling of the sequestered cargo [6].

Autophagy is induced in response to a variety of stress conditions. In cells starved for nutrients or growth factors, autophagy produces critical nutrients and energy that enhance cell survival through the breakdown of cytosolic components. Recent studies demonstrate that other stresses, including hypoxia, growth factor withdrawal, and ER stress, also induce autophagy to enhance cell survival [10–12]. Although autophagy is classically recognized as an adaptive response, basal levels of autophagy are required to maintain homeostasis by preventing the build-up of damaged proteins and organelles. The importance of this basal level of autophagy is poignantly illustrated through studies of mice lacking critical ATGs in neurons, where autophagy deficiency induces the progressive accumulation of protein aggregates, eventually leading to neurodegeneration [13,14].

Although autophagy is vital for maintaining cell survival, excessive autophagy results in programmed cell death, termed autophagic or type 2 cell death [15]. In *Drosophila*, enforced ATG1 expression induces excessive autophagy, resulting in apoptosis [16]. Similarly, destruction of the fly larval salivary gland involves massive autophagy and ATGs are required for this developmental cell death [17]. The dual roles of autophagy in cell survival and death likely play important roles in the pathogenesis of numerous human diseases including neurodegeneration, aging, viral and bacterial pathogenesis, and cancer [6,15].

Autophagy During ECM Detachment

Studies of hypoosmotic swelling were the first to link integrins to autophagy. Upon hypoosmotic swelling in rat liver, cell volume is reduced in part by pathways that inhibit autophagic proteolysis. Inhibiting integrin engagement using an RGD-containing peptide antagonist prevents this reduction in autophagic proteolysis typically seen in hypoosmotic conditions [18,19]. These studies delineated an inverse role between integrin engagement and autophagy induction. In contrast, decreased autophagosome formation is observed in starved prostate epithelial cells (PECs) upon inhibiting integrin function. Addition of function blocking antibodies directed against $\alpha 3$ integrin result in decreased autophagosome formation in starved PECs suggesting ECM attachment is required to maintain autophagy [20]. However, in this study, autophagy levels are assessed by measuring GFP-LC3 puncta, which does not account for autophagosome maturation and turnover in the lysosome. Therefore, reduced punctate GFP-LC3 observed during integrin blockade of starving PECs may represent enhanced autophagosome turnover in the lysosome, rather than decreased bona fide autophagy [21].

Studies of lumen formation in glandular epithelial structures (acini) grown using three-dimensional (3D) culture models have also provided insight into detachment-induced autophagy. In a 3D culture system using MCF10A mammary epithelial cells, lumen formation involves the selective anoikis of central cells lacking ECM contact. Interestingly, although apoptosis is the major pathway to clear cells in the lumen, anti-apoptotic protein overexpression does not prevent hollow lumen formation, suggesting other death pathways compensate when classical apoptosis is inhibited. Electron microscopy demonstrates numerous autophagic vacuoles in the central ECM-detached cells prior to their clearance [22]. Follow-up work corroborates that the lack of integrin engagement directly induces autophagy. Autophagy is rapidly induced in a variety of nontransformed epithelial cell types grown in low attachment

conditions, and has been observed during the detachment of a breast cancer cell line [23,24]. Furthermore, the incubation of attached cells with function blocking antibodies against $\beta 1$ integrin is sufficient to induce autophagy, whereas exogenous addition of a laminin-rich basement membrane to suspended cells inhibits autophagy. Thus, detachment-induced autophagy directly results from the loss of ECM-integrin engagement [23].

Although excessive self-eating was originally proposed to promote type 2 cell death in the 3D lumen, recent data indicates that autophagy enhances survival during anoikis [22]. RNAi downregulation of ATGs promotes apoptosis and decreases clonogenic viability during anoikis, and increases luminal apoptosis during 3D morphogenesis [23]. Similarly, when E1A transformed mouse mammary cells lacking one allele of ATG6 (Beclin 1) are grown in 3D culture, they exhibit accelerated lumen formation compared to wild-type controls [25].

Autophagy also contributes to the clearance of inner cells during embryoid body (EB) cavitation [26]. The preponderance of autophagic vacuoles in the inner cells of EBs, which lack ECM contact, is consistent with the loss of attachment promoting autophagy. However, unlike studies of acinar lumen formation, increased apoptosis is not found in embryoid bodies derived from cells lacking *atg5* or *beclin1*; instead, dead cell corpses fail to clear during cavitation, because autophagy is critical for the presentation of engulfment signals that mediate the phagocytic clearance of apoptotic cells [26]. These phenotypic differences may arise because ATGs (and by inference, autophagy) are completely eliminated in the EB system, whereas they are only partially reduced with RNAi in MCF10A acini. Overall, the studies described above have begun to delineate the biological functions of autophagy upon loss of cell-matrix contact.

Signaling Between the ECM and the Autophagic Machinery

The pathways linking loss of integrin engagement at the cell surface to the autophagy machinery remain elusive. Below, we discuss several intracellular signaling pathways that may connect changes in integrin engagement to autophagy induction, focusing on pathways previously shown to respond to the lack of ECM attachment as well as to mediate autophagy in response to other stresses. Although these pathways overlap and combinations of these pathways may be required for induction of detachment-induced autophagy, they have been grouped into three categories: 1) growth factor and nutrient-sensing pathways; 2) energy-sensing pathways; and 3) integrated stress response (Figure 2).

Growth factor and nutrient-sensing pathways

The proper function of growth factor receptors on the cell surface requires integrin-mediated cell adhesion [1]. For example, EGFR is downregulated upon loss of integrin engagement in multiple epithelial cell types [27]. Downregulation of growth factor receptors or nutrient sensors on the cell surface leads to the inactivation of multiple growth promoting pathways, notably, the mammalian target of rapamycin (mTOR) pathway, which is the archetypal inhibitor of autophagy. Accordingly, mTOR downregulation is also observed following ECM detachment [28].

Since nutrient or growth factor withdrawal induce autophagy, decreased growth factor activation following loss of integrin engagement may explain increased autophagy during suspension. However, enforced epidermal growth factor receptor (EGFR) expression in mammary epithelial cells during cell suspension, which can sustain the activation of key downstream signals in detached cells, does not inhibit detachment-induced autophagy [23, 27]. This indicates detachment-induced autophagy does not result from reduced growth factor signaling, or that other growth factor receptor or nutrient-regulated signals are primary mediators of this response. Further studies are required to dissect if reduced growth factor or

nutrient signaling contributes to detachment-induced autophagy. Remarkably, mTOR activity may be directly controlled in response to cell-matrix adhesion through focal adhesion kinase (FAK). FAK, a critical component of adhesion mediated signaling, can tyrosine phosphorylate TSC2, an upstream mTOR regulator, to suppress its activity and maintain mTOR activation. Inhibiting FAK activity via expression of a kinase dead mutant results in mTOR inhibition [28]. This pathway may provide a more direct link between the loss of integrin function and autophagy.

Energy-sensing pathways

During times of bioenergetic stress, autophagy provides nutrients to cell through degradation products released from the lysosome. Presumably, these basic components can be recycled and used to synthesize new proteins and to provide inputs for energy cycles that produce ATP. In fact, in response to diverse stressors, autophagy inhibition profoundly reduces intracellular ATP levels [10,11,25,26]. Decreased energy (ATP) can be monitored in part by AMP-activated protein kinase (AMPK), which is activated due to increased AMP to ATP ratios via the upstream kinase, LKB1. Upon activation, AMPK phosphorylates and activates the tuberous sclerosis complex (TSC1/2 complex) resulting in downstream inhibition of mTOR. mTOR inhibition not only limits pro-growth signals, but also induces autophagy, which in turn, provides ATP through the recycling of degradation products [29]. Recently AMPK was demonstrated to activate autophagy in response to both LKB1 activation and upon changes in intracellular Ca^{2+} levels leading to activation of the upstream kinase Ca^{2+} /calmodulin-dependent kinase kinase- β (CaMKK β) [30,31]. Although no data demonstrates that the loss of integrin engagement leads to reduced energy (ATP) levels, AMPK is robustly activated during ECM detachment [23]. Whether AMPK activation occurs through a classic LKB1-mediated ATP-sensing pathway or via Ca^{2+} /CaMKK β activation remains unclear. Interestingly, AMPK remains activated in detached cells overexpressing either EGFR or Bcl-2 suggesting this stress pathway is induced during ECM detachment, regardless of the cell's ability to sustain growth factor signaling or prevent apoptosis [23].

Integrated stress response

Phosphorylation of eukaryotic initiation factor 2 alpha (eIF2 α) on serine 51 is a critical convergent point in the integrated stress response (ISR), a general stress-response program conserved from yeast to mammals. This phosphorylation is a central mechanism in stress-induced translational suppression [32]. Accordingly, ECM detachment activates programs that suppress protein translation, and recent work supports that phosphorylated eIF2 α plays a critical role in this suppression [33–35]. Mammary epithelial cells grown in suspension or treated with β 1 integrin blocking antibodies exhibit increased eIF2 α phosphorylation, which depends on the endoplasmic reticulum kinase PERK, a known upstream eIF2 α kinase [35]. Although PERK is classically activated following ER stress to trigger translational repression and apoptosis, during ECM detachment, PERK activation is not a secondary response to ER stress, as several classical ER-stress induced genes are not upregulated [35]. Notably, ER stress, induced via chemicals or the expression of toxic polyglutamine repeat proteins, elicits autophagosome formation that depends on both PERK activation and eIF2 α phosphorylation. [12]. Further studies using cells deficient in components of this pathway will elucidate if the ISR is responsible for autophagy induction during ECM detachment.

The aforementioned pathways represent potential mechanisms a cell may use to link ECM detachment to autophagy induction. Fortunately, several compounds and genetically altered cells lines exist to manipulate various components of these pathways, making it feasible to dissect the intracellular signals required for detachment-induced autophagy.

Detachment-induced autophagy and tumor cell dormancy

Interest in manipulating autophagy to treat cancer has rapidly intensified [36,37]. However, the exact roles for autophagy during cancer progression remain unclear [38]. Early studies of autophagy in cancer support a tumor suppressive role, because a critical autophagy gene, ATG6/Beclin 1, is monoallelically deleted in a large percent of human prostate, ovarian, and breast cancers [39]. Conversely, studies of anoikis suggest autophagy may be a previously unrecognized mechanism to enhance the survival of tumor cells lacking proper ECM contact. One aspect of tumor development where autophagy may play a major role is during tumor cell dormancy. The majority of cancer deaths result from the outgrowth of metastatic lesions at distant sites from the original tumor. These secondary tumors often occur several years following treatment or removal of the primary tumor, and unfortunately, they are often refractory to therapy. Disseminated tumor cells are thought to remain in a dormant state at distant sites for many years before they begin to develop into secondary tumors [40].

Since dormant disease can exist as just one, or a small number of cells, the mechanisms that induce and maintain cellular dormancy are difficult to study experimentally. Recently, both *in vitro* and mouse studies have identified the disruption of $\beta 1$ integrin function as a unique activator of cellular dormancy [40,41]. Inhibition of $\beta 1$ integrin activity prevents tumor cell proliferation, but not cell viability, leading to the induction of a dormant state [42,43]. Further studies indicate that dormancy is mediated downstream of $\beta 1$ integrin through decreases in FAK and MAPK signaling and activation of p38 and eIF2 α [42,44,45]. Although it remains unclear if these mechanisms regulate dormancy in patients, it is proposed that dormancy is induced in disseminated tumor cells exposed to untoward microenvironments that do not facilitate proper ECM-integrin engagement. Notably, the stress pathways induced during dormancy also activate autophagy in other contexts highlighted in the previous section. Accordingly, one can speculate that detachment-induced autophagy in disseminated tumor cells may be vital for maintaining a dormant state or promoting the survival of dormant cancer cells. Two fascinating topics for future investigation include examining the regulation of autophagy in existing models of dormancy as well as interrogating if genetic disruption of ATGs restricts cellular dormancy or initiates the death of dormant tumor cells.

Conclusions

Since ECM engagement of integrins is essential for numerous cellular functions, the regulation of autophagy by cell-matrix contact is perhaps not surprising. Nonetheless, dissecting the intracellular signals that promote detachment-induced autophagy and further defining the biological roles of detachment-induced autophagy during mouse development and carcinoma progression *in vivo* will further illuminate this previously unrecognized aspect of cell adhesion receptor biology.

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Figure 1. Overview of autophagy machinery

The formation and expansion of the early autophagosomes requires two highly conserved ubiquitin-like conjugation systems involving several ATGs [9]. The first conjugation system involves the conjugation of ATG12, an ubiquitin like protein, to ATG5. Mediating this conjugation is an E1 like enzyme, ATG7 that activates ATG12 and passes it to an E2-like conjugating enzyme ATG10. ATG12 is then passed to ATG5 to form a stable conjugate. Disruption of this complex, through the elimination of either ATG5 or ATG12, prevents autophagy induction; thus, knockout or knockdown of these components is often experimentally used to inhibit autophagy. The second conjugation system fuses yeast ATG8 (called LC3 in mammals) to a lipid, phosphatidylethanolamine (PE). The same E1-like enzyme, ATG7 and a distinct E2-like enzyme, ATG3, mediates this lipidation. Recent biochemical evidence indicates that the ATG12:5 complex possesses a E3-like activity required for the efficient PE-lipidation of ATG8 [46,47]. The lipid modification of ATG8/LC3 causes it to relocate to autophagosome membrane, serving as useful autophagy marker; indeed, monitoring ATG8/LC3 relocation to puncta or measuring the levels of PE-modified LC3 (LC3-II) using immunoblotting are two methods commonly used to measure autophagosome formation [21].

Figure 2. Candidate intracellular signaling pathways linking the ECM to autophagy

The loss of integrin engagement at the cell surface may induce autophagy through three potential signaling pathways: growth factor and nutrient-sensing pathways (blue), energy-sensing pathways (green), or the integrated stress response (red). A loss in integrin-mediated adhesion elicits the downregulation of growth factor receptors and/or the reduced activation of downstream signals. Normally, these signals activate mTOR, an established suppressor of autophagy. Furthermore, disruption of cell-matrix contact can trigger both energy-sensing pathways and the integrated stress response through currently unknown mechanisms. This leads to activation of AMPK and $\text{eIF2}\alpha$, both of which are known to stimulate autophagy in other contexts. Further details are provided in the main text.