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Autophagy as a cell death and tumor suppressor mechanism

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Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double or multimembrane autophagic vesicles, and their delivery to and subsequent degradation by the cell's own lysosomal system. Autophagy has multiple physiological functions in multicellular organisms, including protein degradation and organelle turnover. Genes and proteins that constitute the basic machinery of the autophagic process were first identified in the yeast system and some of their mammalian orthologues have been characterized as well. Increasing lines of evidence indicate that these molecular mechanisms may be recruited by an alternative, caspase-independent form of programmed cell death, named autophagic type II cell death. In some settings, autophagy and apoptosis seem to be interconnected positively or negatively, introducing the concept of 'molecular switches' between them. Additionally, mitochondria may be central organelles integrating the two types of cell death. Malignant transformation is frequently associated with suppression of autophagy. The recent implication of tumor suppressors like Beclin 1, DAP-kinase and PTEN in autophagic pathways indicates a causative role for autophagy deficiencies in cancer formation. Autophagic cell death induction by some anticancer agents underlines the potential utility of its induction as a new cancer treatment modality.
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Introduction

The term 'apoptosis' has long been used as a synonym for programmed cell death since the seminal article of Kerr *et al*. published in 1972 (Kerr *et al*., 1972). In their article, the authors classified cell death into two groups. Apoptosis signified the genetically controlled programmed cell death, and the term necrosis described the passive or accidental type of cell death. Since then, the apoptosis field has profited from the increasing attention of the scientific community and a tremendous amount of information has accumulated regarding the molecular mechanisms governing this phenomenon. Although ultrastructural data suggesting the existence of alternative types of programmed cell death have been available since the 1960s, the wider cell death classification proposed by Schweichel and Merker (1973), based on observations which they made in embryonic and fetal tissues of rats and mice, was generally ignored in the scientific literature. Their classification was revisited and broadened by Clarke (1990), resulting in the classification of cell death into four morphological categories as described below.

Type I cell death corresponds to apoptotic cell death, which proceeds by cell shrinkage, chromatin condensation, nucleosomal DNA degradation and fragmentation of the cell into so-called 'apoptotic bodies'. Activation of the caspase family of cysteine proteases gives rise to these characteristic morphological features of apoptosis. The remnants of the cell are removed by the lysosomes of professional phagocytes or neighboring cells after heterophagocytosis.

Type II cell death or autophagic cell death is characterized by the appearance of double- or multiple-membrane cytoplasmic vesicles engulfing bulk cytoplasm and/or cytoplasmic organelles such as mitochondria and endoplasmic reticulum. Autophagic vesicles and their contents are destroyed by the lysosomal system of the same cell. It has been suggested that the overall autophagic activity, in a cell doomed to die under these circumstances, is far more extensive than that associated with the normal cytoplasmic and organelle turnover occurring in healthy cells. As a consequence, the cell 'cannibalizes itself' from inside (autophagy = self-eating in Greek).

Type III cell death is defined as nonlysosomal vesiculate degradation. This mode of death can be further subdivided into type IIIA (nonlysosomal degradation) and type IIIB (cytoplasmic type of degeneration). Sporadic examples of cell death with features of more than one of the cell death morphologies described above also exist (Clarke, 1990).

This review will be dedicated to the molecular basis of autophagy and its possible implications in programmed cell death and in malignant cell transformation. On the one hand, as mentioned above, morphological hallmarks of the autophagy process are prominent during developmental cell death, or in hormone-deprived tissues, where tissue areas or organs are removed *in toto*. This may suggest that the basic machinery of autophagic vesicle formation is recruited to eliminate

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cells in these cases. Yet, on the other hand, as in every complex process in biology, there may exist alternative explanations to interpret the increased occurrence of autophagic vesicles in development and under various physiological and stress conditions. Autophagy is the major cellular route for degrading long-lived proteins and cytoplasmic organelles, and the catabolic advantage of increased autophagy may be critical in various stress conditions. Thus, the process of induced autophagy may rather reflect an adaptive mechanism that attempts to rescue cells from their death destiny, in analogy to the yeast system. In *S. cerevisiae*, autophagy is activated under conditions of nutrient deficiency, providing amino acids and macromolecules necessary for survival under stress conditions. The autophagosomes deliver their content to the vacuole, the degradative organelle in the yeast, for breakdown and recycling (Huang and Klionsky, 2002). So the question that arises is whether type II autophagic cell death actually exists or whether this biological process exclusively reflects a protective mechanism required for continual cell survival. This important issue will be discussed further in this review.

Another important issue relates to the implications of autophagy in malignant transformation. Cancer develops when molecular pathways that control cellular proliferation and/or programmed cell death are subjected to genetic deregulations. The contribution of loss of type I apoptotic responses to cancer formation is now well established and has been extensively analysed at the molecular level over recent years (Hanahan and Weinberg, 2000; Lowe and Lin, 2000; Green and Evan, 2002). Interestingly, a tight correlation between reduced autophagy and cancer has been documented in the past, and recent work indicates that several proteins and pathways that are related to autophagy signaling are being deregulated during malignant transformation, resulting in reduced autophagic activity. In other words, the downregulation of autophagic activity in malignant cells suggests that a failure in autophagy signaling may be instrumental in cancer formation, and that autophagy may function under certain circumstances as a safeguard mechanism that restricts uncontrolled cell growth. Yet, the mechanisms by which the reduced autophagy contributes to cancer etiology could be interpreted in different ways. They may reflect the advantage provided by the breakdown of type II autophagic cell death in cases where this process operates to eliminate cells when necessary. Alternatively, reduced autophagy may increase the proliferative capacity of cells by different mechanisms as discussed below.

Another point to be considered with respect to autophagy/cancer relationship is the observation that some malignant cell types respond to anticancer agents by triggering autophagy, indicating the potential utility of autophagic cell death induction in cancer therapy. The present review aims at summarizing these accumulating data concerning autophagy signaling and the role of changes in autophagic capacity and of autophagic cell death in cancer formation.

The basic machinery of autophagy

In autophagy, cytoplasmic constituents, including organelles such as the mitochondria, are first enwrapped by a membrane sac. Although endoplasmic reticulum, Golgi or a less well-characterized membrane compartment called phagophore have been proposed as origins of autophagosome membranes, recent studies indicate that autophagic vesicles may be formed *de novo* through nucleation, assembly and elongation of small membrane structures (see Figure 1a) (Noda *et al*., 2002). Closure of these membranes results in the formation of double membrane structures called autophagosomes. After a few steps, autolysosomes are generated by fusion of the outer membranes of the autophagosomes and late endosomes or lysosomes. Lysosomal hydrolases degrade the cytoplasm-derived contents of the autophagosome together with its inner membrane (Figure 1a). The molecular mechanisms governing the formation of autophagic vesicles were initially uncovered using the yeast *Saccharomyces cerevisiae* as a model system (Ohsumi, 2001; Huang and Klionsky, 2002). These studies resulted in the cloning by different laboratories of sets of Apg, Aut and Cvt genes that partially overlap, which are finally named in the unified nomenclature as 'Atg' (autophagy-related) genes (Tsukada and Ohsumi, 1993; Thumm *et al*., 1994; Harding *et al*., 1995; see

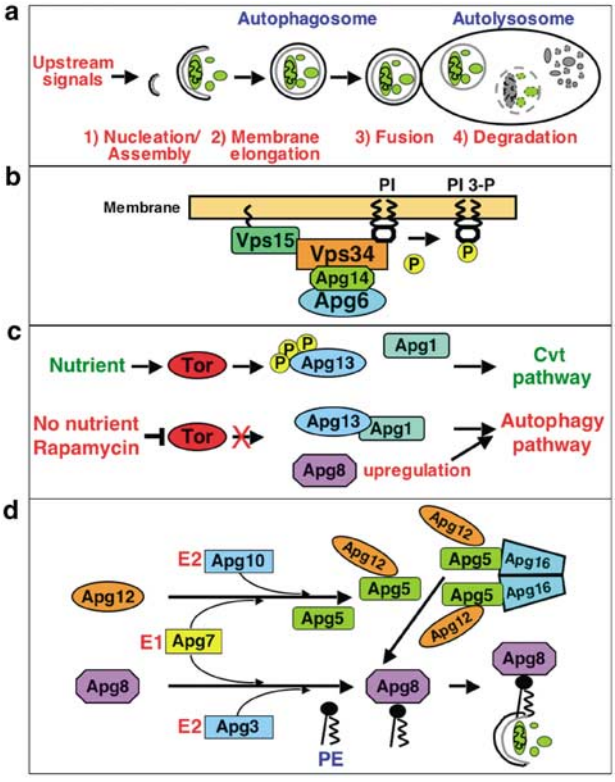

Figure 1 Mechanisms involved in autophagic vesicle formation. (**a**) General scheme of stages of autophagic vesicle formation and degradation. (**b**) Phosphatidylinositol 3-kinase complex and Phosphatidylinositol 3-phosphate (PI 3-P) generation from phosphatidylinositol (PI). (**c**) Autophagy induction by Tor inhibition. (**d**) Ubiquitin-like conjugation systems involved in autophagic vesicle formation. PE: phosphatidylethanolamine

Klionsky *et al*., 2003, for the unified nomenclature). Of note, the Cvt pathway is involved in the delivery of resident hydrolases to the vacuole in yeast, thus, unlike autophagy this biosynthetic pathway is active under nonstarved conditions, and so far in mammalian cells, no pathway equivalent to the Cvt pathway has been reported. Mammalian orthologues of some of the autophagy-related genes have been cloned, and their function is indeed preserved in the mammalian system (Mizushima *et al*., 2003b). For a detailed review of the yeast and mammalian basic autophagy machinery, the reader is referred to the following excellent reviews (Ohsumi, 2001; Huang and Klionsky, 2002; Mizushima *et al*., 2002).

In this review, we will briefly relate to various components of the basic machinery on which some exiting information has been recently accumulated, and then mainly focus on those proteins and signaling molecules that may couple the autophagy pathways to type II programmed cell death and/or to cancer. Although the molecular information is still fragmented and far from being completed, some biochemical pathways start to emerge and those will be briefly described. In addition, two general characteristics of autophagy are worthwhile mentioning at this stage, since they will be instrumental in the subsequent discussions. That is, the notion that the process of autophagy depends on both continuous protein synthesis and continuous presence of ATP (Kim and Klionsky, 2000). These general characteristics of the system are relevant in particular when the hallmarks of autophagic cell death will be analysed.

Phosphatidylinositol 3-kinase (PI 3-kinase) complex

The type of PI 3-kinase involved in autophagy signaling is class III PI 3-kinase and its product phosphatidylinositol 3-phosphate (PI 3-P) (Petiot *et al*., 2000). Autophagosome precursors are not generated in cells treated with PI 3-kinase inhibitors wortmannin and 3-methyladenine (3-MA), indicating that PI 3-kinase activity is important in the early stages of autophagic vesicle formation (Mizushima *et al*., 2001). In fact, 3-MA has been widely used as a general inhibitor of autophagy for decades (Seglen and Gordon, 1982).

In the yeast, a PI 3-kinase complex consisting of Apg6 (also named Vps30), Apg14, myristylated serine kinase Vps15 and the PI 3-kinase Vps34 functions in autophagic vesicle formation (Figure 1b). Apg14 serves as an adaptor for Apg6–Vps34 interactions (Kim *et al*., 2002). Vps15 kinase regulates Vps34 activity (Stack *et al*., 1995). At least in the yeast system, Vps15 and Vps34 may be part of another complex involved in the Cvt pathway; therefore, Apg14–Apg6 association confers autophagy specificity to the PI 3-kinase complex (Kihara *et al*., 2001b). Proteins containing FYVE or PX motifs are able to interact with PI 3-P, thus the generation of this lipid derivative by PI 3-kinase may lead to autophagy through recruitment of some proteins containing these domains and necessary for the formation of the autophagic vesicle (Gillooly *et al*., 2001; Wishart *et al*., 2001). A significant breakthrough in the discovery of the mammalian counterpart of this system occurred with the identification of Beclin 1 as the mammalian orthologue of Apg6 (Liang *et al*., 1999). Beclin 1 expression in MCF7 breast cancer cells increased the number of autophagic vesicles and the degradation of long-lived proteins upon amino-acid starvation. Furthermore, overexpression of Beclin 1 was able to complement the defective autophagic activity in Apg6 disrupted yeast. In addition, although the mammalian Apg14 has not yet been cloned, Beclin 1, and a p150 myristylated protein kinase interact with the mammalian orthologue of Vps34 PI 3-kinase, indicating that the PI 3-kinase complex may also be functional in the mammalian system (Panaretou *et al*., 1997; Kihara *et al*., 2001a). In line with its role in PI 3-kinase signaling, addition of 3-MA is able to prevent autophagy induction by Beclin 1 in MCF7 cells (Liang *et al*., 1999).

In the yeast, autophagy-related proteins accumulate in one or a few perivacuolar structures called preautophagosomal structure (PAS), which are suggested to function as organizing centers of autophagosome formation. So far, no PAS-like structure has been identified in the mammalian system. Nevertheless, the localization of Beclin 1/PI 3-kinase to the trans-Golgi network in mammalian cells suggests that this compartment may be important in mammalian autophagic vesicle formation (Kihara *et al*., 2001a).

Tor kinase and Apg 1 complex

Tor kinase is believed to be a gatekeeper for initiation of the autophagic pathway since it acts as a sensor for amino acids and ATP. Additionally, in mammalian cells, Tor kinase is also an integrator of growth factor-induced signals (like insulin or insulin-like growth factor receptor signals) to autophagic pathway, through its connection with Akt pathway. Yeast Tor protein kinase was discovered as the target of the antifungal drug rapamycin. Treatment of cells with rapamycin inhibits Tor activity and induces autophagy in both yeast and mammalian cells. Along this line, phosphorylation of proteins in the Tor pathway (e.g. p70S6K, and its target the ribosomal protein S6) coincides with inhibition of autophagy in mammalian cells (Blommaart *et al*., 1997). Furthermore, inhibition of the Tor pathway leads to transcriptional upregulation of the Apg8 gene (Kirisako *et al*., 1999; Huang *et al*., 2000), an essential component of the autophagic vesicle formation/size expansion machinery (see below).

The mechanisms by which Tor protein regulates autophagy have been mainly studied in the yeast system. It was found that Tor phosphorylates Apg13 under nutrient-rich conditions. In its hyperphosphorylated form, Apg13 has a low affinity for Apg1, leading to a reduced Apg1 kinase activity. (Kamada *et al*., 2000; Scott *et al*., 2000). Under these conditions, Cvt pathway is activated (Figure 1c). In contrast, when Tor activity is blocked, Apg13 is rapidly dephosphorylated and tightly associates with Apg1 kinase. This promotes the activation of Apg1 leading to autophagy induction (Figure 1c).

The role of the catalytic activity of Apg1 in the induction of autophagy is still controversial. Although the Apg1 kinase activity is upregulated during autophagy, a non-kinase related role for Apg1 was documented for the induction of autophagosomes (Kamada *et al*., 2000; Abeliovich *et al*., 2003). The targets of Apg1 kinase and the relevance of the mammalian homologue of Apg1, named Ulk1, in autophagy signaling are yet to be discovered.

Ubiquitin-like conjugation systems involved in membrane elongation

Two ubiquitin-like conjugation systems are instrumental in autophagic vesicle biogenesis. The first consists of the Apg12 system in which Apg12 is conjugated to Apg 5 in a ubiquitination-like manner (Mizushima *et al*., 1998). The conjugation reaction starts by the activation of Apg12 by an E1-like enzyme called Apg7. This is followed by the transfer of Apg12 by Apg7 to an E2-like enzyme called Apg10 (Shintani *et al*., 1999; Tanida *et al*., 1999). Finally, Apg12 is conjugated to Apg5 by the covalent linkage of a carboxyterminal glycine of Apg12 to a lysine residue in the central part of the Apg5 protein (Figure 1d). Apg5 is the only known target of Apg12. Apg12/Apg5 conjugation reaction occurs just after its synthesis and almost all Apg12 molecules in a cell are found in a conjugated form (Kuma *et al*., 2002). Therefore, Apg12/Apg5 conjugation is constitutive and is not influenced by autophagy-inducing stimuli such as amino-acid starvation.

The Apg12/Apg5 conjugation is functional in the formation and stabilization of a larger protein complex, containing Apg12/Apg5 and Apg16 (Figure 1d) (Mizushima *et al*., 1999, 2003a). Apg16 (Apg16L in mammalians) has the capacity to homo-oligomerize through its coiled coil domain. Thus, the Apg12/Apg5/Apg16 complex forms larger protein complexes (of 350 kDa in yeast and 800 kDa in mammals) through oligomerization of its Apg16 component. The Apg12/Apg5/Apg16 complex localizes to the outer membrane of the forming autophagosome and it dissociates following completion of the autophagosome (Mizushima *et al*., 2001, 2003a).

The second ubiquitin-like protein is Apg8, and its target is surprisingly a lipid molecule, phosphatidylethanolamine (PE) (Ichimura *et al*., 2000). For this conjugation reaction to occur, the Apg8 protein has to be processed by the cleavage of a portion of its carboxy-terminus at the time of its translation. The protease responsible for this processing reaction, the Apg4 protein, is interestingly a cysteine aspartase like the caspases involved in apoptosis (Kirisako *et al*., 2000). Cleavage of Apg8 by Apg4 exposes a carboxy-terminal glycine. The E1-like protein Apg7, which is also responsible for Apg12 activation, activates Apg8 as well. The E2-like protein Apg3, on the other hand, is specific for Apg8 conjugation (Ichimura *et al*., 2000). Finally, the carboxy-terminal glycine of Apg8 protein is conjugated via an amide bond to an amino group of PE (Figure 1d). This conjugation causes the otherwise cytoplasmic/peripherally membrane-bound form of Apg8, to associate tightly with membranes (Kirisako *et al*., 1999). The lipidation reaction is reversible and Apg4 protease, also acting like a ubiquitin deconjugating enzyme, is capable of cleaving the amide bond between Apg8 and PE, enabling the recycling of Apg8.

The yeast Apg8 has several mammalian orthologues and isoforms (Mann and Hammarback, 1994; Chen *et al*., 2000; Kabeya *et al*., 2000; Sagiv *et al*., 2000). Yet, only MAP1LC3 (microtubule-associated protein 1 light chain 3, or shortly LC3) is involved in autophagic vesicle formation, suggesting that LC3 is the functional homologue of Apg8 in mammals. LC3 has been recently proposed to serve as a marker for autophagic vesicles and autophagic activity, based on the observation that its autophagic 'membrane recruited' form displays a different intracellular localization and increased electrophoretic mobility on SDS polyacrylamide gels (Kamada *et al*., 2000). The contribution of the other isoforms to autophagic mechanisms remains to be characterized.

Interestingly, the two conjugation systems, Apg12 and Apg8, may be coordinated by the joint requirement for Apg7. Furthermore in yeast deficient for Apg12 or Apg5, membrane recruitment of Apg8 does not occur (Suzuki *et al*., 2001). Similarly, in mammalian systems, $Apg5^{-/-}$ ES cells are defective in autophagosome formation and Apg12 conjugation to Apg5 is necessary for the membrane targeting of LC3 (Mizushima *et al*., 2001). The interdependence between the two ubiquitin-like conjugation systems has been documented thoroughly in mammalian cells (Tanida *et al*., 2001, 2002).

Signaling pathways and other regulators of autophagy

G proteins

A constitutive autophagic pathway is operational in the colon cancer cell line HT-29 and heterotrimeric G proteins localized to Golgi/ER membranes are involved in its regulation (Houri *et al*., 1993; Ogier-Denis *et al*., 1995). The GDP bound form of the Gαi3 subunit is the autophagy-inducing form in this system (Ogier-Denis *et al*., 1995). Hydrolysis of GTP to GDP by a GTPase-activating protein GAIP stimulated autophagy, as did expression of AGS3, a protein that stabilizes the GDP-bound form of the Gαi3 (Ogier-Denis *et al*., 1997; Pattingre *et al*., 2003b).

GAIP was, in turn, shown to be regulated by the Ras/Raf-1/ERK1/2 signaling pathway. Activated Ras induced autophagy in HT-29 cells, in conjunction with activation of Raf-1, ERK1/2 and GAIP (Pattingre *et al*., 2003a). ERK1/2 was capable of directly phosphorylating GAIP *in vitro*, thereby stimulating its GTPase activity towards Gαi3, leading consequently to autophagy (Ogier-Denis *et al*., 2000). This pathway is sensitive to amino acids. Activated Raf-1 failed to induce autophagy in the presence of amino acids (Pattingre *et al*., 2003a). In their absence, ERK1/2 was phosphorylated and exhibited increased activity towards GIAP. Thus, the presence of amino acids interferes with the

activation of GAIP by ERK1/2. Furthermore, Raf-1 undergoes an amino-acid-dependent inhibitory phosphorylation. Thus, autophagy is suppressed by the presence of amino acids through downregulation of the Raf-1/ERK1/2/GAIP pathway.

This signaling pathway can also be modulated by Akt. For example, EGF leads to the activation of both ERK1/2 and Akt. Under these circumstances, autophagy and GAIP activation failed to be induced despite the activation of ERK1/2 since Akt led to the inactivation of Raf-1 by phosphorylation (Pattingre *et al*., 2003a). In fact, PTEN, which is a negative regulator of Akt, stimulated autophagy in HT-29 cells (Arico *et al*., 2001). Thus, the cross talk between the Ras/Raf-1/ERK1/2 and the Akt signaling pathways seems to regulate the outcome of autophagy-inducing signals.

PKR/eIF2α

The regulation of initiation of protein translation is an issue that attracted much interest in recent years. Major efforts were concentrated on the phosphorylation of the eucaryotic initiation factor 2α (eIF2α) on serine-51 by a conserved family of protein kinases, a process that represents a central mechanism in stress-induced translation regulation. GCN2 is the yeast eIF2α kinase and the mammalian family consists of four eIF2α kinases, one of which is the double-stranded RNA-dependent protein kinase (PKR). The phosphorylation on serine 51 reduces the global protein translation in cells. A recent study showed that eIF2α phosphorylation induced autophagy in yeast and in primary murine embryonic fibroblasts (Talloczy *et al*., 2002). In yeast, eIF2α phosphorylation by the GCN2 was necessary for starvation-induced autophagy but not for rapamycin-induced autophagy. Therefore, eIF2α pathway acts upstream to or independently from the Tor pathway. While phosphorylated eIF2α reduces protein translation, it has been found that a general translational inhibitor such as cycloheximide did not induce autophagy, indicating that autophagy is not simply a result of a global arrest in translation. It was found in this respect that the translation of a few mRNAs continues in a selective manner. For example, the translation of GCN4, which is a transcriptional transactivator of starvation-induced genes, increases when eIF2α is phosphorylated concomitant with global inhibition in translation. GCN4 has been shown to activate the transcription of some key Apg genes in autophagy regulation (e.g. Apg1, Apg14), thus mechanistically linking eIF2α phosphorylation to induction of autophagy (Natarajan *et al*., 2001).

In mouse primary embryonic fibroblasts, PKR was essential for autophagy induction in response to herpes simplex-1 virus infection (Talloczy *et al*., 2002). eIF2α phosphorylation was also necessary for amino acid/serum starvation-induced autophagy in primary fibroblasts. Therefore, in mammalian cells, eIF2α phosphorylation regulates autophagy in response to stresses like nutrient starvation and viral infection. Of note, in the mammalian system, internal ribosome entry site (IRES)-dependent protein translation was also upregulated by eIF2α phosphorylation during amino-acid starvation, thus increasing the profile of proteins that continue to be translated by eIF2α phosphorylation to trigger the autophagy pathway (Fernandez *et al*., 2002).

Death-associated protein kinase (DAP-kinase or DAPk) and DAPk-related protein-1 (DRP-1)

The DAPk family of kinases is a novel mammalian regulator of autophagy. DAPk is a calcium/calmodulin-regulated serine/threonine kinase associated with actin cytoskeleton. Several lines of research identified this kinase as a positive mediator of cell death in response to various stimuli such as inferferon-γ, activation of Fas receptors, TNF-α, TGF-β and detachment from the extracellular matrix (Cohen and Kimchi, 2001; Jang *et al*., 2002). DAPk belongs to a family of highly related death associated kinases, which includes DRP-1 and ZIP-kinase (Kogel *et al*., 2001). DRP-1 is the closest homologue to DAPk since it shares high identity both in the catalytic and the calcium/calmodulin binding domains. Interestingly, overexpression of each of the two kinases in carcinoma cell lines (which lack functional p53) induced different hallmarks of type II cell death including autophagic activity (Figure 2) (Inbal *et al*., 2002). In addition, both DAPk and DRP-1 were found to be necessary for the induction of autophagy. A dominant-negative construct of DRP-1 reduced the level of starvation and tamoxifen-induced autophagy of MCF-7 breast carcinoma cells, while reduction of DAPk expression by antisense RNA attenuated interferon-γ-induced autophagy in HeLa cells. Interestingly, overexpressed DRP-1 localized to the lumen of the autophagic vesicles. This raises the possibility that DRP-1 may have a direct role in autophagic vesicle formation, possibly by phosphorylation of components of autophagic vesicle formation machinery (Inbal *et al*., 2002).

BNIP3 and HSpin1

Overexpression of BNIP3, a hypoxia-inducible BH3-like domain containing protein, led to autophagic type II cell death in cancer cell lines (Vande Velde *et al*., 2000). BNIP3 integration to the mitochondrial outer membrane induced permeability transition pore opening, and inhibitors of pore opening blocked cell death. Moreover, overexpression of Bcl-2, which was previously shown to interact with BNIP3 protein, attenuated BNIP3-induced cell death. Interestingly, cytochrome *c* release or AIF nuclear translocation was not observed upon BNIP3 overexpression.

HSpin1 is the human orthologue of *Drosophila* Spin, a transmembrane protein that is necessary for programmed cell death in *Drosophila* reproductive and nervous systems. The overexpression of HSpin1 in cancer cell lines induced caspase-independent, necrotic-like cell death and it led to an increase in MDC-labeled vesicles (MDC – monodansylcadaverin – is a marker of

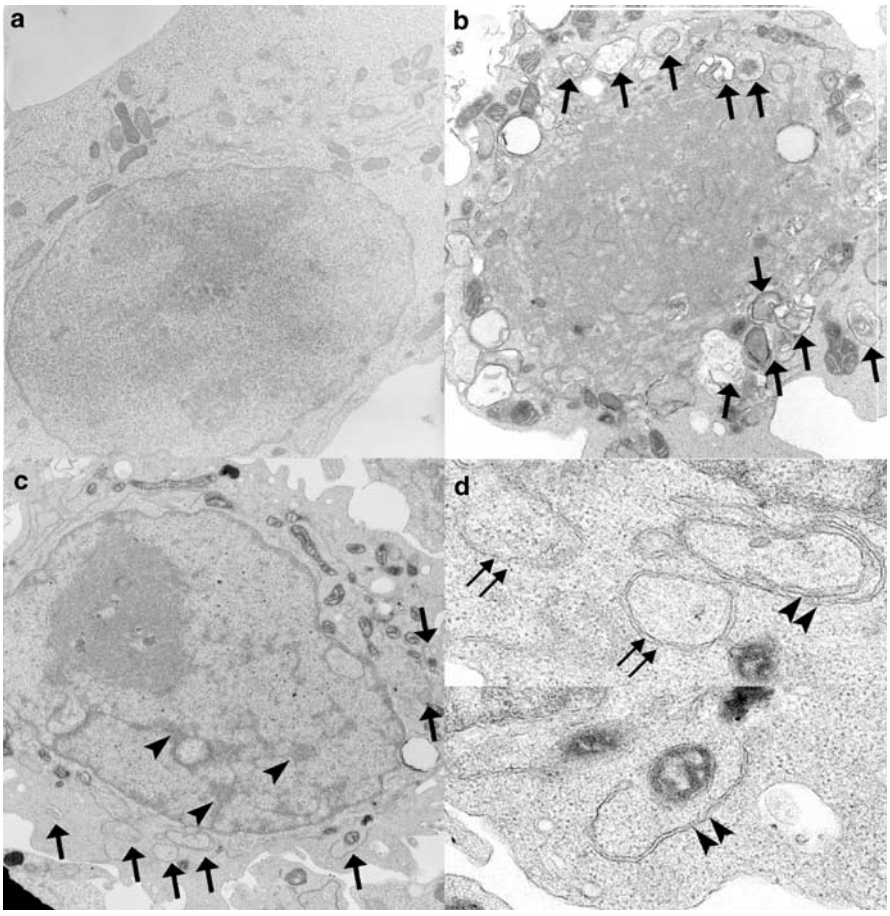

Figure 2 Transmission electron micrographs of DAP-kinase and DRP-1-induced autophagic type II cell death. In all, 293 cells transfected with luciferase (**a**), an activated DRP-1 (DRP-1 Δ73) (**b**) or activated DAP-kinase (DAPkΔCaM) (**c**) constructs. Arrows indicate autophagic vesicles and arrowheads indicate partially condensed chromatin. (**d**) Selected regions of the micrographs with typical autophagic vesicles containing cytoplasmic material (double arrows), organelles like endoplasmic reticulum or mitochondrion (double arrowheads) are enlarged

acidic compartments including autophagosomes/autolysosomes), suggesting that the cell death may have an autophagic nature (Yanagisawa *et al*., 2003). HSpin1 also contains a BH3-like domain and it interacted with Bcl-2 and Bcl-XL in TNF-α treated cells. Furthermore, overexpression of Bcl-XL inhibited HSpin1-induced cell death.

Autophagy: a survival or death pathway?

The very existence of autophagic cell death is a matter of debate in different scientific communities. As mentioned above, the presence of autophagic vesicles in dying cells may reflect the existence of autophagic type II cell death, or alternatively may be an adaptive response to maintain continual cell survival under stress conditions. Is autophagy causative to the demise of the cells or is it part of a rescue mechanism activated during cellular distress? Are the two possibilities mutually exclusive or can both apply, depending on the severity of the response and influences by other signaling pathways?

A few independent lines of research support the survival functions of autophagy. First, since autophagy provides the cell with amino acids and other building blocks when these macromolecules are not available from extracellular sources, autophagic response may be an adaptive response connected to the survival of the cell under unfavorable conditions. Similar mechanisms are functional in both yeast and mammalian cells and they can even occur at the organism level as well (Blommaart *et al*., 1997). Liver, for example, constitutes an extreme case in which amino-acid deprivation-induced autophagy provides building blocks for biosynthetic pathways for the entire organism. It should also be stressed that autophagy is an important physiological process that plays a role in the turnover and elimination of excess or damaged organelles like peroxisomes, mitochondria and endoplasmic reticulum. The observation that depolarized mitochondria are rapidly eliminated by autophagy in primary hepatocytes led Lemasters and co-workers to propose that autophagy may also be a protective mechanism against apoptosis, increasing the threshold of mitochondria-dependent cell death (Elmore *et al*., 2001). According to this model, elimination of damaged mitochondria by autophagy would prevent the release of proapoptotic substances from mitochondria, thus preventing apoptosis

(Lemasters *et al*., 1998). In the absence of such 'clean up', the release of molecules like cytochrome *c* and AIF from damaged mitochondria would lead to apoptosis and in case of extreme damage and ATP depletion, to necrotic cell death.

Another point of view referring to the survival function of autophagy during developmental stages was further discussed by Kitanaka and Kuchino (1999). The occurrence of developmental autophagy in a given tissue and at a given time could be a secondary effect of the programmed destruction of blood vessels feeding that tissue. Thus, autophagic vesicles in the tissue would result from starvation after ischemia and not from direct death signals to that tissue. In fact, in addition to nutrient starvation, hypoxia is also able to induce autophagy in certain cell types (David *et al*., 1992). According to this model, programmed cell death in neighboring tissue would control autophagy only indirectly and the appearance of autophagic vesicles in a developmental stage-specific manner would be coincidental.

In the light of the above said, are there other scenarios where autophagy is rather causative to cell death and how mechanistically autophagy could impose on cells a 'point of no return'? The characterization of programmed cell death that lacks hallmarks of type I cell death, yet displays autophagic activity that is much intensive than that observed during the starvation response supports this concept. Examples are death of palatal epithelial cells during closure, regression of the Mullerian duct during male sexual development, death of the central cells of the intestine during cavity formation and hormone deprivation-induced regression of mammary gland or atrophy of prostate. The reader is referred to the following articles for a complete list (Schweichel and Merker, 1973; Clarke, 1990; Bursch, 2001). Furthermore, autophagy and apoptosis can be simultaneously observed in the same tissue, indicating that different types of cell death may develop concomitantly to accomplish the important tasks of tissue remodeling and other developmental processes that require genetically controlled programmed cell death (Clarke, 1990; Bursch, 2001). At the cellular level, it has been shown that in some systems the total area of autophagic vacuoles and dense bodies may roughly be equal to, or greater than, that of cytosol and organelles outside the autophagic vesicles (Clarke, 1990). Therefore, an autophagic activity at this level should be capable of destroying major proportions of the cytosol and organelles finally leading to the total collapse of the cellular functions and to an irreversible type of cellular atrophy. In line with this, Xue *et al*. (2001) showed that in superior cervical ganglia neurons, HeLa or CHO cells treated with a cell death-inducer in the presence of caspase inhibitors, the majority of the mitochondria were destroyed by autophagy and cells became smaller and they were eventually irreversibly committed to death. Moreover, the finding that the autophagy inhibitor, 3-MA, was able to prevent the death of 24-h amino-acid starved primary hepatocytes further supports the hypothesis that the complex mechanisms of autophagic vesicle formation mentioned above are the direct causes of cell death (Schwarze and Seglen, 1985). Unlike type I apoptosis, in this type of cell death caspases are not activated, and therefore DNA degradation is not pronounced, the nucleus stays intact until the late phases of cell death and cellular fragmentation is not observed (see Table 1). Membrane blebbing on the other hand seems to be a common feature of apoptosis and autophagic cell death. Thus, the occurrence of

Table 1 Comparison of the two types of programmed cell death

	Type I apoptotic	*Type II autophagic*
Nucleus	Chromatin condensation Pyknosis of nucleus DNA laddering and nuclear fragmentation	Partial chromatin condensation Sometimes pyknosis of nucleus Nucleus intact until late stages No DNA laddering
Cytoplasm	Cytoplasmic condensation Ribosome loss from RER Fragmentation to apoptotic bodies Lysosomal protease release to cytosol may be involved Mitochondrial permeability transition is often involved Caspases are active	Increased autophagic vesicle number Increased autolysosome number Increased lysosomal activity Enlarged Golgi, sometimes dilatation of ER Mitochondrial permeability transition may be involved Caspase-independent
Cell membrane	Blebbing	Blebbing
Corps clearence	Heterophagy by other cells	Late and occasional heterophagy by other cells
Detection methods	Electron microscopy Nuclear/cellular fragmenation detection Caspase activation tests Caspase substrate cleavage tests DNA laddering detection TUNEL staining Increase in sub G1 cell population assessed by FACS analysis Annexin V staining	Electron microscopy Test of increased long-lived protein degradation Tests of increased lysosomal activity (MDC, acridine orange or lysotracker staining, etc) Test of increased cytoplasmic sequestration (LDH or sucrose sequestration tests) Detection of LC3 recruitment to autophagic membranes (protein band shift or change in intracellular localization)

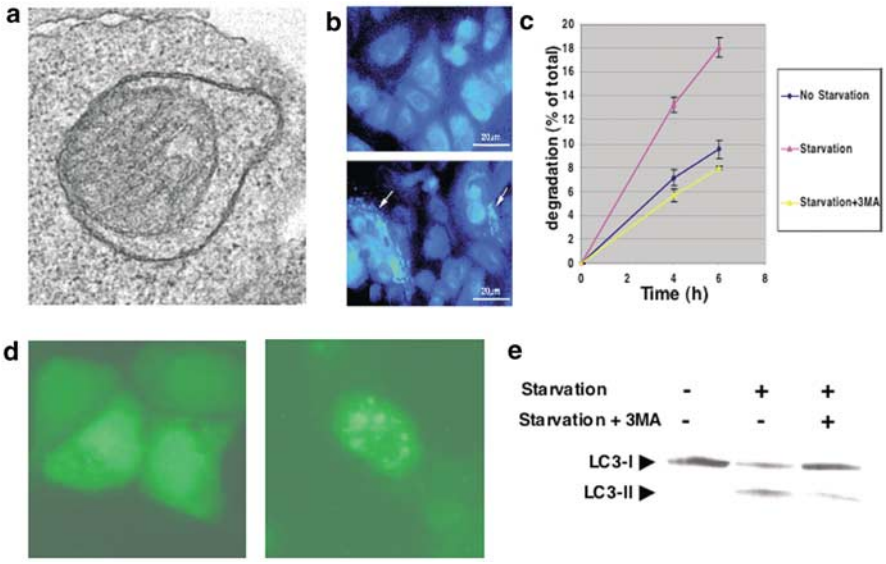

Figure 3 Examples of autophagic activity detection methods. (**a**) transmission electron microscopy of an autophagic vesicle containing a mitochondrion in a DRP-1 transfected 293 cell. (**b**) MDC staining of luciferase transfected (top) or DRP-1 transfected (bottom, white arrows) autophagic MCF-7 cells. (**c**) Measurement of radiolabeled long-lived protein degradation in 4 and 6 h amino-acid/serum starved murine primary hepatocytes. 3-MA addition blocks autophagic degradation. (**d**) GFP-LC3 intracellular localization in luciferase transfected (left) or DAPk transfected (right) 293 cells. (**e**) Electrophoretic mobility change of LC3 from nonautophagic (LC3-I) form to autophagic membrane recruited (LC3-II) form upon 2 h amino-acid/serum starvation of murine embryonic fibroblasts. Inhibition of autophagy by 3-MA prevents LC-II formation

caspase-independent cell death concomitant with an increased autophagic activity may be indicative of autophagic type II cell death. To demonstrate more directly autophagic cell death, several tests detecting autophagic activity should be combined with a more precise collection of type II cell death morphological characteristics, which still awaits precise definition, ATP dependence and lack of classical hallmarks of apoptosis. The demonstration of the autophagic vesicles by electron microscopy is still the 'golden standard' for assessing the autophagic activity (Figure 3a). Increased autophagic/lysosomal activity may also be demonstrated by MDC staining (Figure 3b) or by the measurement of the degradation rate of radiolabeled long-lived proteins (Figure 3c). The change in the intracellular localization of LC3 protein (Figure 3d) and its increased electrophoretic mobility (Figure 3e) upon LC3 recruitment to autophagic membranes provide the first molecular marker-based methods for the detection of autophagic activity. The molecular characterization of the basic autophagic machinery discussed above will lead to the development of additional sophisticated tools for the direct biochemical assessment of the autophagic activity in dying cells.

Autophagy–apoptosis connection

Are autophagy and apoptosis mutually exclusive mechanisms? Data from several studies point out that apoptosis and autophagy may be interconnected in some settings, and in some cases even simultaneously regulated by the same trigger resulting in different cellular outcomes as discussed below. Recent data supporting the interconnection between the two processes came from gene expression profiles during steroid-triggered developmental cell death in the *Drosophila* system where several apoptosis-related genes are upregulated together with autophagy-related genes (Gorski *et al*., 2003; Lee *et al*., 2003). Apoptosis/autophagy interaction may manifest itself in various ways. Depending on the cellular context and stimulus, autophagy may be indispensable for apoptosis by preceding and further turning on apoptosis. In other settings, autophagy may rather antagonize/delay apoptosis, and there are also examples where the two processes may be mutually exclusive acting as backup mechanisms of each other for imposing irreversible cell death.

In some experimental systems, execution of apoptosis is preceded by and even depends on the occurrence of autophagy (Figure 4a). In these settings, autophagy inhibitors like 3-MA delay apoptosis while conversely, broad-range caspase inhibitors fail to inhibit autophagy. One well-studied example is the primary sympathetic neurons, exposed to neural growth factor deprivation or to c-Ara treatment. These cells displayed a 30-fold increase in autophagic activity followed by DNA degradation and other hallmarks of apoptosis. The latter could be inhibited by 3-MA at the stage of cytochrome *c* release and caspase activation. Conversely, the pan-caspase inhibitor Boc-Asp(OMe)fmk did not affect the induced autophagic activity and the cells eventually died in a caspase-independent manner (Xue *et al*., 1999). Another example is the TNF-α-induced apoptosis of T-lymphoblastic leukemia cell lines

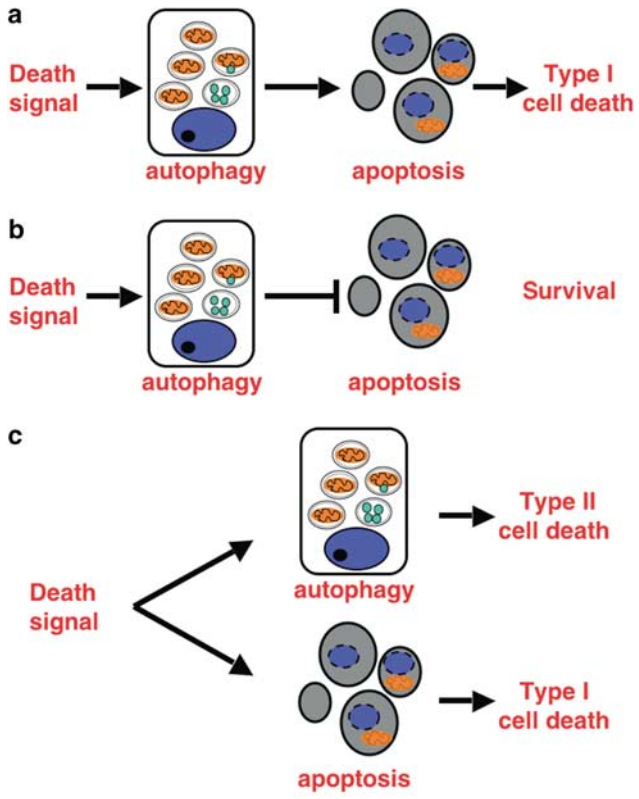

Figure 4 Apoptosis/autophagy connection in programmed cell death context. (**a**) Autophagy may be indispensable for apoptosis occurrence. (**b**) Autophagy may antagonize apoptosis (**c**) Apoptosis and autophagy may occur independent of each other. Inhibition of apoptosis may convert cell death morphology to autophagic and *vice versa*

(Jia *et al*., 1997). In this case, however, while autophagy induction was essential, it was not sufficient by itself to induce apoptosis, indicating the necessity of additional independent death signals provided by the TNF-α for obtaining cell death responses (Jia *et al*., 1997).

In other cellular settings, autophagy may antagonize apoptosis and inhibition of autophagy may increase the sensitivity of the cells to apoptotic signals (Figure 4b). A well-described example is the case of sulindac sulfide (a nonsteroidal anti-inflammatory drug)-induced apoptosis in HT-29 colon carcinoma cells (Bauvy *et al*., 2001). In this system, type I apoptotic cell death was accelerated in an HT-29 mutant clone, which displays decreased autophagic capacity due to overexpression of a GTPase deficient mutant of Gαi3 protein. Consistent with this line, treatment of the parental HT-29 cells with autophagy inhibitors increased their sensitivity to apoptosis (Bauvy *et al*., 2001). Since cytochrome *c* release was faster in the HT-29 mutant clones, the authors suggested that elimination of damaged mitochondria by autophagy may be a mechanism of delaying apoptosis in this system.

Apoptosis and autophagy may also manifest themselves in a mutually exclusive manner (Figure 4c). Inhibition of autophagic activity in cells may switch responses to death signals from autophagic type II programmed cell death to apoptotic type I cell death. For example, various malignant glioma cell lines were reported to exert type II autophagic cell death in response to arsenic trioxide (Kanzawa *et al*., 2003). Inhibition of autophagy in these cells by exposing them to bafilomycin A1 (an H^+-ATPase inhibitor blocking autophagy by inhibiting autophagic vesicle acidification) led to cell death by apoptosis instead. In the opposite example, once type I apoptosis in neurons, HeLa or CHO cells was blocked by a broad-range caspase inhibitor, a selective elimination of mitochondria by autophagy took place instead, resulting in an irreversible commitment to cell death (Xue *et al*., 2001). It is possible that some re-evaluation with specific autophagic markers of previously reported settings in which inhibition of caspases was found to convert apoptotic cell death to a 'necrotic-like' cell death, may provide additional examples of a switch between the two types of cell death (see Kitanaka and Kuchino, 1999 for examples).

The mechanisms underlying this switch between apoptosis and autophagy are currently obscure. The striking fact that proteins like DAPk are capable of inducing both apoptosis and autophagy depending on cellular settings suggests that they may act as molecular switches or integrators of the two types of programmed cell death. Additionally, increasing number of reports show a direct physical interaction between autophagy-inducing proteins and proteins involved in apoptosis (especially antiapoptotic Bcl-2 family members). The list includes the Apg6 orthologue Beclin 1, and proteins containing a BH3-like domain such as BNIP3 and HSpin1 (Liang *et al*., 1998; Vande Velde *et al*., 2000; Yanagisawa *et al*., 2003). Although in some cases, Bcl-2 or Bcl-XL overexpression protected cells from autophagic cell death, the role of these protein–protein interactions in apoptotic or autophagic cell death context still needs to be clarified (Vande Velde *et al*., 2000; Xue *et al*., 2001; Yanagisawa *et al*., 2003).

In fact, Bcl-2 family of proteins are functional in apoptosis, which is based on mitochondrial damage and this event was also proposed to be a signal for the activation of autophagy (Elmore *et al*., 2001). It is, therefore, likely that mitochondria are involved in the integration of apoptotic and autophagic cell death. According to the model proposed by Lemasters *et al*., autophagy may block apoptosis by preventing the release of proapoptotic mitochondrial factors to the cytoplasm due to the elimination of damaged mitochondria (Lemasters *et al*., 1998). The signal conveyed from damaged mitochondria to stimulate autophagy may involve mTor since a fraction of this protein was recently found associated to the mitochondrial outer membrane where it sensed osmotic stress-induced mitochondrial dysfunction (Desai *et al*., 2002). Mitochondrial damage-induced autophagic cell death may occur especially under conditions where mitochondrial-based apoptosis is not dominant or it is blocked by caspase inhibitors. In support of this idea, in a recent review, Piacentini *et al*. (2003) proposed that, in neurons, the prothymosin-α that inhibits the apoptosome formation may serve as a switch to determine the mode of cell death. According to their model, in case

prothymosin-α levels are high enough to block mitochondrial-based apoptosis, cell death will switch to autophagic type, whereas low levels will allow cells to die by apoptosis. Yet, direct experimental evidence about the role of protymosin-α in autophagy induction is still missing.

Autophagy and cancer

Autophagic capacity and cellular transformation

During the late 1970s and throughout the 1980s, an inverse relationship between autophagic activity and malignant transformation has been established. It was found in this respect that the autophagic capacity of cancer cell lines was often lower than their normal counterparts and failed to be increased in response to serum/amino-acid deprivation or high cell density. For example, protein degradation rates in transformed fibroblasts were lower than their nontransformed counterparts, especially under nutrient and/or serum starvation conditions (Gunn *et al*., 1977; Lockwood and Minassian, 1982; Cockle and Dean, 1984; Gronostajski and Pardee, 1984; Knecht *et al*., 1984). This fitted the more general observations that the rate of cell growth is influenced by a balance between the rates of protein synthesis and autophagic degradation of long-lived proteins, the latter being significantly lower in proliferating cells than in stationary cells (Hendil, 1977; Otsuka and Moskowitz, 1978; Pfeifer and Dunker, 1985; Papadopoulos and Pfeifer, 1987; Pfeifer *et al*., 1988; Tessitore *et al*., 1988; Lee *et al*., 1992).

A decrease in autophagic capacity was also observed during animal experimental carcinogenesis. Cells from preneoplastic liver nodules, or primary hepatocellular carcinomas induced by chemical carcinogens, had a decreased autophagic capacity compared to normal cells from the liver (Schwarze and Seglen, 1985; Canuto *et al*., 1993; Kisen *et al*., 1993). Furthermore, autophagic vesicles isolated from liver nodules and hepatocellular carcinomas showed lower activities of lysosomal enzymes (Ahlberg *et al*., 1987; Yucel *et al*., 1989). In culture, cells isolated from nodules survived much better in the absence of amino acids compared to normal hepatocytes (Schwarze and Seglen, 1985). In the light of these data, Schwarze and Seglen (1985) proposed that the survival advantage offered by downregulation of autophagy may result from the improved protein balance obtained by preventing excessive protein loss upon starvation. *In vivo*, maintenance of this positive protein balance by decreased autophagy may offer a clonal advantage to the preneoplastic cells, compared with normal hepatocytes that face starvation-related stress due to meal-related fluctuations of amino-acid concentrations.

Of note, studies on carcinogen-induced pancreatic cancer in rats showed that during tumor progression, cells from premalignant nodules and adenomas had a paradoxically increased autophagic capacity, especially in response to the autophagy inducer, vinblastine (Rez *et al*., 1999; Toth *et al*., 2002). These cells also had an increased capacity of autophagic vesicle degradation. Yet, in contrast to premalignant cells or adenoma cells, pancreatic adenocarcinoma cells had decreased autophagic activity even in response to vinblastine (Toth *et al*., 2002). Therefore, autophagic capacity first increases during premalignant stages of pancreatic carcinogenesis, and then decreases during pancreatic adenoma to adenocarcinoma transition, suggesting again that decreased autophagic activity is necessary and/or compatible with malignant stages of pancreatic cancer.

Obviously, all these studies raised the possibility that the breakdown of the autophagy process may contribute to the development of cancer. Yet, direct causal relationships were missing at this stage, and it was clear that once the genes that control/execute autophagy will be identified, they will provide the appropriate genetic tools to assess directly the role of autophagy deficiencies in cancer development.

Genetic deregulations that link loss of autophagic capacity to cancer development

Recent reports show that some previously known oncogenes and tumor suppressor genes impinge upon autophagic pathways in addition to their other well-known functions. Thus, some steps in the malignant transformation could result from the deregulation of these autophagy-related aspects in their mode of action. The PTEN tumor suppressor gene, class I PI 3-kinase and Akt oncogenes, Ras and Myc oncogenes fall into this category. A second category is exemplified by DAPk. Unlike the previously mentioned genes, this gene was first identified via its cell death-promoting functions and only later different lines of research, including human tumor screens, established its role as a tumor suppressor gene. Since induction of autophagy was recently identified as one of the intrinsic activities of DAPk, the frequent loss of DAPk expression in human tumors provides another possible link between autophagy and cancer. The third and most straightforward category consists of genes that constitute the downstream autophagy 'execution' genes. In case some of them may turn out to be '*bona fide*' tumor suppressor genes, subjected to loss or inactivation in cancer, then autophagy deficiencies may clearly be part of the multistep process of tumorigenicity. Beclin 1, the mammalian orthologue of Apg6, which is a component of the PI 3-kinase complex involved in autophagic vesicle nucleation (Figure 1b), provides the first example of this important third category of genes coupling autophagy to cancer. This section will summarize the current information on autophagy-related oncoproteins and tumor suppressors.

c-Myc oncogene

Under conditions where caspases are blocked by specific inhibitors, ectopic expression of c-Myc induced a 'necrotic-like' cell death in serum-starved fibroblasts (McCarthy *et al*., 1997). Later on, re-examination of

similar systems proved that overexpression of c-Myc in rat fibroblasts caused an increase in autophagic activity (Tsuneoka *et al*., 2003). In this system, a mutant c-Myc defective in proapoptotic and oncogenic transformation activities still induced autophagy, suggesting that the autophagy-induction domain of c-Myc is different from its apoptosis and transformation-inducing domains. These data indicate that, in addition to its growth-promoting and proapoptotic activity, c-Myc can turn on autophagy and may be autophagic type II cell death, as a safeguard mechanism that may further limit tumor growth.

Ras oncogene

Another oncoprotein, Ha-Ras, induced autophagic cell death in glioma and gastric cancer cell lines (Chi *et al*., 1999). Cell death was p53-independent and it was not blocked by Bcl-2 overexpression. K-Ras and N-Ras also exerted this nonapoptotic cell death-inducing activity, indicating that autophagy induction may be a common characteristic of Ras oncoprotein family. Ras-induced apoptosis and senescence are important mechanisms to counteract Ras-induced transformation. Autophagic cell death-induction by Ras proteins may constitute another level of control against cancer formation. In support of this, increased Ras expression was observed in areas of spontaneous, nonapoptotic neuroblastoma degeneration, which manifested characteristics of autophagic cell death (Kitanaka *et al*., 2002). The fact that these areas are also found near fibrovascular stroma suggests that autophagy is not a secondary effect of ischemia, but rather plays a direct causative role. In line with this, overexpression of Ha-Ras also induced autophagic cell death in neuroblastoma cell lines *in vitro*. Similarly, an activated form of Ha-Ras induced autophagy in HT-29 colon cancer cells and the Raf-1/ERK 1/2 pathway was involved in this phenomenon (Pattingre *et al*., 2003a).

PTEN, PI 3-kinase and the Akt pathway

Loss of function mutations of PTEN gene as well as increased expression of both class I PI 3-kinase and Akt are frequent events in human tumors. The tumor suppressor PTEN, which is an inhibitor of the PI 3-kinase/Akt pathway has been recently shown to promote autophagy in HT-29 colon cancer cells (Arico *et al*., 2001). PTEN phosphoinositide phosphatase activity blocked the Akt survival pathway and induced autophagy. Consistent with this, a constitutively active form of Akt inhibited autophagic activity in these cells (Arico *et al*., 2001). Activation of the Akt pathway switches on survival pathways, which lead to the inhibition of apoptosis and activates mTor protein, which antagonizes autophagy. Moreover, the protein products of Tsc1 and Tsc2 tumor suppressor genes responsible for the tuberous sclerosis syndrome, hamartin and tuberin, have recently been shown to inhibit mTor signaling and Akt was able to relieve this inhibition through phosphorylation and inactivation of hamartin (Inoki *et al*., 2002; Tee *et al*., 2002). It is highly probable that tuberin and hamartin induce autophagy in mammalian cells through mTor pathway inhibition. Therefore, activation of Akt pathway and Tor signaling, and loss of PTEN tumor suppressor may contribute to malignant transformation by the simultaneous inhibition of apoptosis and autophagic cell death.

DAPk and cancer

The current notion that DAPk functions as a tumor suppressor gene emerged from three independent lines of research. The first relates to large screens of human tumors for the status of the DAPk gene, performed over the last 3 years by many laboratories. The initial step in this direction was the seminal observation that DAPk expression was lost in several cell lines derived from B cell neoplasms, and bladder, breast and renal cell carcinomas (Kissil *et al*., 1997). The treatment of some of these cell lines with 5′-azadeoxycytidine restored the expression of DAPk, demonstrating that the loss of expression was often due to the methylation of the DAPk promoter region. Gene silencing by methylation is a well-documented mechanism in tumorigenesis. Alternative mechanisms of DAPk inactivation, such as deletions, were also documented (Simpson *et al*., 2002).

Several studies from different laboratories analysed DAP-kinase gene methylation in human primary tumors, mainly using methylation-specific PCR assays. When tested, the hypermethylation of DAPk correlated well with the loss of DAPk mRNA and protein expression. DAPk gene methylation was observed in a major fraction of lymphomas, lung, breast, ovarian, colon, cervix, bladder, head and neck, brain and prostate cancers (Raveh and Kimchi, 2001). Two independent studies showed that DAP-kinase promoter methylation in non-small-cell lung cancer is associated with aggressive disease and poor survival (Tang *et al*., 2000; Kim *et al*., 2001). DAP-kinase hypermethylation positive cases showed advanced pathological stage, larger tumors and lymph node involvement. Similarly, DAPk hypermethylation in head and neck cancer, oligodendrogliomas, pituitary tumors and bladder cancers correlated with tumor aggressiveness (Sanchez-Cespedes *et al*., 2000; Lehmann *et al*., 2002; Simpson *et al*., 2002; Tada *et al*., 2002; Alonso *et al*., 2003). The above data indicate that DAP-kinase may have a tumor suppressor and metastasis suppressor function.

The other research lines that further established the role of DAPk in the suppression of tumor growth and metastasis rely on experimental systems performed in cell cultures and mouse model systems. One of the directions was tested in an experimental model of lung metastasis (Inbal *et al*., 1997). DAP-kinase expression was lost in high metastatic variants of two lung cancer cell lines tested, although it was still expressed in the low metastatic variants. Reintroduction of DAPk by stable expression to the highly metastatic clones attenuated the metastatic capacities of these cells. Primary tumor growth was also delayed and was further attributed to increased cell death, reflecting an augmentation of the

sensitivity of the tumor cells expressing DAPk to death-inducing insults. Thus, DAPk acts as a tumor suppressor and a metastasis suppressor protein by increasing the sensitivity of tumor cells to programmed cell death during growth at the primary site and during the multistep process of metastasis.

A second experimental system consisted of primary embryonic fibroblasts, where activated DAPk suppressed c-Myc and E2F1-induced transformation by activating p53-dependent apoptosis (Raveh *et al*., 2001). This event was dependent on the presence of the p53 regulator p19ARF protein. DAPk-null mouse embryonic fibroblasts showed a reduced p53 induction in response to oncogenes. The reduction in p53-induction correlated well with the relative resistance of the DAPk-null fibroblasts to cell death induced by oncogenes. Therefore, attenuation of the p53-dependent proapoptotic signals, induced by oncogenes like c-Myc due to the inactivation or loss of DAPk protein, may be an important factor contributing to the oncogenic transformation *in vivo*. The involvement of DAPk in c-Myc-induced autophagy needs to be tested.

Induction of p53-dependent apoptosis by DAPk indirectly, by inhibiting integrin signaling in an inside-out manner, has been recently suggested as an alternative mechanism of p53-induction by DAPk (Wang *et al*., 2002). DAPk has also been shown to induce p53-independent apoptosis in some experimental systems, but the relevance and the importance of these pathways to cancer initiation and progression remain to be explored (Jang *et al*., 2002).

The role of autophagy in the tumor and metastasis suppressor activity of DAPk is currently unknown. This issue can be explored *in vivo* by the use of DAPk knockout mice model. We are currently analysing the effect of collaboration between oncogenes and loss of DAPk by crossing DAPk knockout mice to cancer-prone mice. Analysis of the autophagic activity and autophagic cell death of the tumor cells that develop in the presence of absence of DAPk will be important in the evaluation of the role of DAPk-mediated autophagic cell death in cancer formation.

Beclin 1 and cancer

The Beclin 1 gene is localized to chromosome 17q21, a locus that is deleted in 75% of ovarian and 50% of breast and 40% of prostate cancers. A screen of 22 breast cancer cell lines by Beclin1-specific FISH analysis showed the presence of Beclin1 allelic deletions in 41% of these cells leading to the classification of Beclin1 as a candidate tumor suppressor gene (Aita *et al*., 1999).

Additional studies showed that Beclin 1 protein expression was lost in eight human breast carcinoma cell lines out of 11 tested (Liang *et al*., 1999). Analysis of Beclin 1 protein expression by immunoblotting of human breast carcinoma samples in comparison with matched normal breast tissue revealed that Beclin 1 protein levels were lower in 15 out of 17 breast cancer cases. Furthermore, a significant decrease in Beclin 1 protein levels was observed in 18 of 32 breast cancer cases by immunohistochemistry, confirming carcinoma cell-specific loss of Beclin 1 expression. Therefore, attenuation of Beclin 1 protein expression is a frequent event in human breast cancer.

At the initial steps, the tumor suppressor properties of Beclin 1 were analysed by *in vitro* clonigenicity assays and by assessing tumorigenicity in nude mice (Liang *et al*., 1999, 2001). Stable transfection of Beclin 1 in MCF7 breast cancer cells reduced the malignant phenotype, slowed down the proliferation rate and reduced their growth in soft agar (Liang *et al*., 1999). Furthermore, the tumorigenic capacity in nude mice of MCF-7 overexpressing the Beclin 1 gene was severely attenuated. The ectopically expressed Beclin 1 co-localized with both intracytoplasmic organelles and nuclei. Interestingly, inhibition of nuclear export by leptomycin B or transfection with Beclin 1 mutant deficient in a CRM1 nucleocytoplasmic export signal resulted in a loss of both the autophagy-inducing and tumor suppressor activities (Liang *et al*., 2001). This finding suggests that the CRM1 nuclear export pathway is important in the regulation of Beclin 1 activity.

Most recently, mice that carry heterozygous disruption of Beclin 1 were independently generated by two laboratories (Yue *et al*., 2003; Qu *et al*., 2003). It was found that this haploinsufficiency caused a high incidence of spontaneous tumors in these Beclin $1^{+/-}$ heterozygous mutant mice. The tumor types were mostly lung adenocarcinomas, hepatocellular carcinomas and lymphomas. They developed between 13 and 22 months of age (incidence corresponded to 30–59 versus 14% in controls). The autophagic activity was significantly reduced in Beclin 1 deficient cells as assessed either *in vivo* in the mutant mice or *in vitro* in Beclin $1^{-/-}$ embryonic stem (ES) cells (Yue *et al*., 2003; Qu *et al*., 2003). Interestingly, no signs of reduced apoptotic cell death could be detected in mice tissues, while the cellular proliferative capacity was significantly increased. Also, the apoptotic responses of Beclin $1^{-/-}$ ES cell cultures to DNA damage or serum withdrawal were normal, suggesting that this gene is not involved in type I apoptotic cell death. Altogether, these studies provide a direct proof that autophagy reduction is causal to tumor formation and establish that Beclin 1 is a haploinsufficient tumor suppressor gene. The contribution of possible loss of type II autophagic cell death to tumorigenesis in Beclin $1^{+/-}$ mice still needs to be explored.

Of note, Beclin 1 was originally isolated in a yeast two-hybrid screen for Bcl-2-interacting protein. The role of the Beclin 1/Bcl-2 interaction in the tumor suppressor and autophagy-inducing functions of Beclin-1 and in the Bcl-2 oncogenic activity remains to be determined.

Autophagic cell death in response to anticancer agents

Autophagy and autophagic cell death can be activated in cancer cell lines in response to various agents used in cancer treatment. The treatment of breast carcinoma cell line MCF-7 with estrogen antagonist tamoxifen caused

cell death with autophagic characteristics (Bursch *et al*., 1996). Estradiol and the autophagy inhibitor 3-MA were able to block death, underlining the active and autophagic nature of the tamoxifen-induced cell death. Similarly, arsenic trioxide treatment of malignant glioma cell lines induced G2/M arrest and autophagic cell death (Kanzawa *et al*., 2003). Radiation treatment induced autophagic cell death in breast, prostate and colon cancer cell lines (Paglin *et al*., 2001). Glioblastoma multiforme cell lines also responded to ionizing radiation by halting cell proliferation and by increasing their autophagic activity (Yao *et al*., 2003). The terminal metabolite of prostaglandin J series with antitumor activities, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, induced autophagic type II cell death in prostate cancer cells (Butler *et al*., 2000). Drugs like vinblastin and rapamycin also induce autophagy under experimental conditions, but the role of autophagic cell death in the anticancer effects of these drugs remains to be explored.

Autophagic cell death inhibition and angiogenesis

According to a recent report, autophagic type II cell death may be a means to inhibit angiogenesis (Chau *et al*., 2003). Stimulation of angiogenesis in solid tumors and their subsequent metastasis is a crucial step in allowing tumors to increase their mass. The angiogenesis inhibitor endostatin induced endothelial cell death, which showed characteristics of autophagic type II cell death, including caspase-independence, increased autophagic activity and sensitivity to 3-MA. Interestingly, PTEN tumor suppressor protein, which induces autophagy, was shown to inhibit tumor neovascularization in a nude mouse orthotopic brain tumor model (Wen *et al*., 2001). The role of autophagic cell death in tumor vascularization inhibition is worth considering.

Conclusions and perspectives

Data accumulated in the last 30 years on the mechanisms and signaling of apoptosis contributed tremendously to our understanding of cancer biology and provided us with potential targets for new strategies of cancer treatment. On the other hand, mechanisms governing the alternative modes of programmed cell death have been neglected for decades. In the last few years, however, increasing efforts have been committed to elucidate the biology of these other forms of cell death. The preparation of this special issue of 'Oncogene' journal is the best indication of this changing point of view.

Thanks to the remarkable work done, especially in yeast, a general picture of the mechanisms governing autophagic vesicle formation and degradation has formed. Searches for the mammalian counterparts of autophagy genes are ongoing in several independent laboratories, and the observation that the basic mechanisms of autophagy are preserved from yeast to humans is encouraging. Characterization of the mammalian proteins has shown that many autophagy-related proteins possess several isoforms, which may be expressed in the same tissue, indicating the level of complexity in the regulation of mammalian autophagy.

How upstream signals regulate autophagy, and how they stimulate autophagic sequestration and degradation machinery, constitute the challenge for future studies. How autophagic cell death recruits autophagic machinery and how the cell switches a stress-response to a suicidal weapon are other issues to be clarified. Current data suggest that mTor is one of the integrator molecules transducing amino acid, growth factor-dependent and mitochondrial stress-related signals to the autophagic machinery. In light of the finding that in some systems, autophagy and apoptosis are interdependent phenomena, it is plausible that there exist molecular switches between the two types of cell death. If so, proteins capable of turning on one type of cell death or another in an experimental system-dependent manner and/or interacting with the regulators of both types of cell death are good candidates for this function. Thus, proteins like DAPk, Beclin 1, BNIP3, HSpin1 or protymosin-α may be part of the network connecting the two types of cell death.

Decrease in autophagic activity in transformed cells, the identification of an autophagy execution gene such as Beclin 1 as a novel tumor suppressor gene and involvement of other well-established oncoproteins and tumor suppressor proteins in autophagy-related pathways indicate that autophagy may play a role in cancer prevention. One way through which breakdown of autophagy could contribute to malignant transformation emerges from the link of this fundamental process to type II programmed cell death. The role of apoptosis in restraining abnormal cell proliferation has been extensively studied, leading to the emergence of apoptosis-based therapies of cancer (Reed, 2002). However, even upon inhibition of apoptosis, cell death may still proceed due to the activation of nonapoptotic pathways (Leist and Jaattela, 2001). In some cases, inhibition of apoptosis might even enhance the activation of the alternative cell death mechanisms (Kitanaka and Kuchino, 1999; Leist and Jaattela, 2001). Accordingly, during malignant transformation, one can hypothesize that inactivation of apoptotic pathways may be accompanied by the inactivation of nonapoptotic programmed cell death pathways like autophagic cell death. In case of Ras and c-Myc, in analogy with the proapoptotic activity of these proliferation-inducing proteins, a third arm emanating from them and leading to autophagic cell death seems to be present. If this arm is functional during cancer formation, overcoming apoptotic mechanisms by the inactivation of apoptotic pathways will not be enough for malignant transformation, and inactivation of alternative death pathways like autophagic cell death pathway should occur in parallel. Or else, considering that there may be common pathways regulating both types of death, molecular changes downregulating both apoptotic and autophagic pathways will be preferential. Overexpression of Bcl-2 or loss of DAPk seen in many types of cancers may constitute examples of this strategy used by cancer cells.

Another contribution of decreased autophagic capacity to malignant transformation could result from its function in protein metabolism and cell size determination. Proliferating cells need to reach minimal size prior to cell division and cancer cells are no exception. More than 90% of the proteins in a cell have a long half-life, and a change in the rate of degradation of these long-lived proteins may dramatically change the protein mass of the cell. Autophagy is the major pathway of degradation for these proteins. Therefore, the rate of autophagy has a determinant role in cell mass modulations, and changes in the autophagic capacity of cells should correlate with their ability to proliferate. Furthermore, some of these long-lived proteins may be involved in the regulation of the cell proliferation and their elimination by autophagy may constitute one of the mechanisms controlling undesired proliferation.

Another contribution of defective autophagy to cancer formation may be related to its role as a guardian of cellular homeostasis. Autophagy contributes to the elimination of cytoplasmic proteins with abnormal conformations and destroys damaged or excessive organelles. Disturbances of autophagy may lead to accumulation of these abnormal proteins and organelles that may perturb normal cellular growth control mechanisms. Accumulating damaged organelles may even cause an increase in the level of genotoxic substances like reactive oxygen species, thus leading to mutations. These hypotheses need to be tested.

Increasing number of studies propose autophagic cell death as the mechanism of action of some anticancer agents. These observations suggest that autophagic cell death induction in cancers may have a therapeutic value. Molecules capable of inducing both autophagic cell death and apoptosis then would have the value of a 'golden bullet' since they will be capable of triggering both caspase-dependent and autophagic caspase-independent cell death at the same time. In this context, endothelial autophagic cell death induction may be clinically relevant, considering the success of the drugs targeting tumor vascularization.

Development of new and practical tests and markers for autophagy detection is one of the most important tasks in the field. Assays based on changes in the intracellular localization and electrophoresis mobility of LC3 upon its recruitment to autophagic membranes are promising methods, and the development of GFP-LC3 mice by Mizushima *et al*. (2003c) offers a valuable tool for the analysis of the role of autophagy in physiological and pathological conditions, in the whole mouse.

Interest in the field of autophagy and autophagic cell death is constantly increasing, suggesting a booming future for this field. Considering the physiological and pathological importance of autophagy pathway, this interest will be most rewarding from both a basic scientific and clinical point of view.

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