# Chapter 1
# Molecular Pathways of Different Types of Cell Death: Many Roads to Death

**Dmitri V. Krysko, Agnieszka Kaczmarek and Peter Vandenabeele**

**Abstract:** Cell death is a fundamental cellular response that has a crucial role in shaping our bodies during development and in regulating tissue homeostasis by eliminating unwanted cells. Three major morphologies of cell death have been described: apoptosis (type I), cell death associated with autophagy (type II) and necrosis (type III). In mammalian cells, the apoptotic response is mediated by either an intrinsic or an extrinsic pathway, depending on the origin of the death stimuli, and is almost always caspase-dependent. For a long time necrosis has been considered to be an accidental and uncontrolled form of cell death. However, evidence is accumulating that necrotic cell death in some cases can be as well controlled and programmed as caspase-dependent apoptosis. Autophagy is foremost a survival mechanism that is activated in cells subjected to nutrient or obligate growth factor deprivation. When cellular stress continues, cell death may continue by autophagy alone, or else it often becomes associated with features of apoptotic or necrotic cell death, depending on the stimulus and cell type. It is debatable whether autophagic cell death is an alternative way of dying, different from apoptotic and necrotic cell death, or whether failure of autophagy to rescue the cell can lead to cell death by either pathway. The aim of this chapter is to provide a general overview of current knowledge on signalling events that result in apoptosis, necrosis and cell death associated with autophagy.

**Keywords:** Apoptosis • Necrosis • Autophagy • Caspases • Mitochondria

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## 1.1 Introduction

Our life begins from a single cell that needs to divide into cells that are destined for different fates to form a complex organism. It is almost paradoxical that millions of cells die during development and later in life. Since ancient times it has been known that some structures of multicellular organisms, such as the fetal arterial duct, is committed to disappear, but the first description of cell death was introduced after the establishment of the cell theory by Carl Vogt in 1842 (Clarke et al. 1995). Over the following years, many scientists described naturally occurring cell death, yet most biologists were interested in understanding the life of the cell rather than its death (Clarke et al. 1996). The concept of programmed cell death (PCD) was introduced in 1965 by Lockshin and Williams (Lockshin et al. 1965) as a process that occurs in predictable places and at predictable times during embryogenesis, pointing to the fact that cells are somehow programmed to die during the development of the organism. Later on Kerr and co-authors (Kerr et al. 1972) described the morphological characteristics of cell death during development and tissue homeostasis and coined the term apoptosis (derived from the Greek word meaning "falling off", as of leaves from a tree) to distinguish this type of cell death from necrosis, the death of cells due to physico-chemical insult. Nowadays in mammals it is possible to discriminate about eleven types of cell death (Melino et al. 2005). In this chapter we will briefly discuss several of these death types, with particular focus on type I (apoptotic cell death), type II (autophagic cell death) and type III (necrotic cell death) (Schweichel et al. 1973).

## 1.2 Apoptosis

Apoptosis (type I cell death) occurs through a sequence of specific morphological changes in the dying cell: condensation of the cytoplasm and margination of the nuclear chromatin into one or several large masses, with subsequent formation of membrane-bound apoptotic bodies, containing a variety of cytoplasmic organelles and nuclear fragments, which are engulfed by neighboring cells and by macrophages (Kerr et al. 1972; Schweichel et al. 1973). Our understanding of the mechanisms involved in apoptosis in mammalian cells was gained from investigations of the programmed cell death that occurs during development of the nematode *C. elegans* (Horvitz 1999). In this organism, 1090 somatic cells are generated during the formation of the adult worm, of which 131 undergo apoptosis or "programmed cell death". These 131 cells die at the same place and time during development, demonstrating the remarkable accuracy of this system. Apoptosis occurs during normal development and ageing as a homeostatic mechanism to maintain cell populations in tissues (Diez-Fraile et al., Chap. 2, this Vol.). The mechanisms of apoptosis are highly complex, involving an energy-dependent cascade of molecular events. To date, research indicates that there are two main pathways to apoptosis. In mam-

malian cells, the apoptotic response is mediated by either the intrinsic or the extrinsic pathway, depending on the death stimulus. Both pathways finally converge on the proteolytic activation of downstream effector caspases. Apoptotic cells exhibit several biochemical modifications, such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition and engulfment. All the other chapters in this book are dedicated mainly to discussing the latest information about the phagocytosis of apoptotic cells.

Although the concept of apoptosis has been introduced 30 years ago, the death pathways that regulate apoptosis have remained elusive until the last decades. In the past years it has become clear that a group of cysteinyl aspartate-specific proteases, named caspases, are central regulators and executioners of apoptotic, inflammatory signalling pathways, cell differentiation and proliferation (Lamkanfi et al. 2007). Capases are widely expressed as inactive proenzymes in most cells and once activated they can often activate other procaspases, allowing initiation of a protease cascade. This proteolytic cascade, in which one caspase can activate others, amplifies the apoptotic signal and thus leads to rapid cell death. Human caspases are broadly categorized into initiators (caspase-2, -8, -9, -10), effectors or executioners (caspase-3, -6, -7), and inflammatory caspases (caspase-1, -4, -5; Cohen 1997; Rai et al. 2005). Inflammatory caspases in mouse include caspase-1, -11 and -12 (Lamkanfi et al. 2002). Caspase-11, which is reported to regulate cytokine maturation during septic shock (Kang et al. 2002), caspase-12, which may mediate endoplasmic-specific apoptosis (discussed later in this chapter; Groenendyk et al. 2005; Nakagawa et al. 2000), and involved in modulation of septic shock (Saleh et al. 2004; Lamkanfi et al. 2005). Caspase-14 in mouse and human is specifically expressed in the skin and is involved in proper functioning of the cornified envelop of the skin (Denecker et al. 2007; Denecker et al. 2008).

### *1.2.1 Extrinsic Pathway of Caspase Activation*

The extrinsic signalling pathways that initiate apoptosis involved transmembrane receptor-mediated interactions. It is triggered at the cell surface by the binding of an extracellular death ligand, such as FasL, tumour necrosis factor (TNF), Apo3-ligand, or TRAIL (TNF-related apoptosis inducing ligand/Apo-2 ligand), to its cell-surface death receptor, such as Fas receptor and tumour necrosis factor-receptor 1, death receptor (DR) 3 (APO-3/TRAMP), DR 4 (TRAIL-R1), DR 5 (TRAIL-R2/TRICK2) and DR 6 (Nagata 1999; Sheikh et al. 2000; Chen et al. 2002). Death receptors typically consist of an extracellular region containing varying numbers of cysteine-rich domains required for ligand binding, and an intracellular region with a death domain (DD) motif for homotypic protein-protein interactions. A typical example for the extrinsic pathway is Fas-induced apoptosis (Fig. 1.1). When Fas ligand or agonistic antibodies bind to the homotrimeric Fas receptor, an apoptotic signal is transduced by the death domain through homotypic interactions; these interactions recruit adaptor molecules also containing a DD, such as FADD (Aravind et al. 1999;

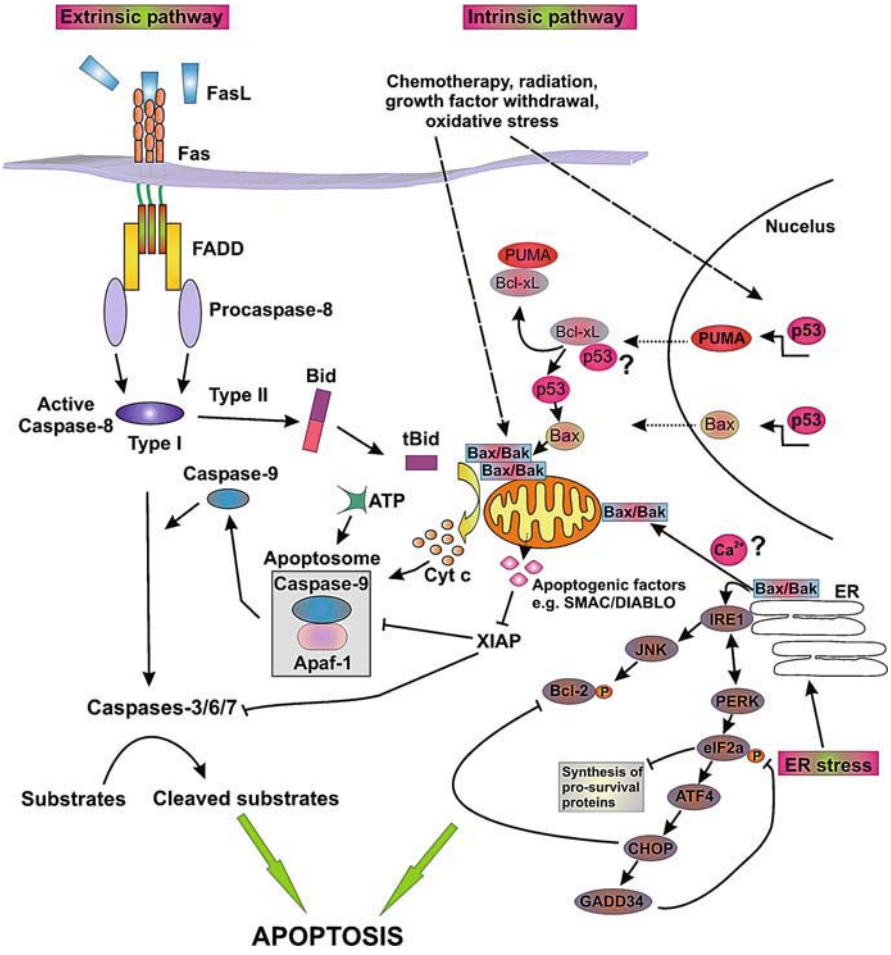


**Fig. 1.1** An overview of apoptotic pathways. The extrinsic pathway involves oligomerization of death receptors by their ligands (e.g. FasL), resulting in recruitment and activation of caspase-8. Caspase-8 can execute apoptosis either directly, though cleavage of caspase-3 (type I), or indirectly (type II), by cleaving Bid, which then translocates to mitochondria to initiate the intrinsic pathway. As an example of the extrinsic pathway, Fas-mediated signalling is shown. The intrinsic pathway is activated when BH3-only proteins cause oligomerization of Bax or Bak, which triggers mitochondrial release of apoptogenic factors such as cytochrome c, SMAC/DIABLO, HtrA2/OMI, and EndoG into the cytosol. In the cytosol each of these proteins participate in a different way in the apoptotic process. Cytochrome c in the presence of ATP triggers apoptosome assembly and activation of the caspase cascade. SMAC/DIABLO and HtrA2/OMI contribute mainly to apoptosis by interfering with the caspase-inhibitory function of the IAP. In addition, to initially proposed role of p53 in the nucleus to trasnactivate genes such as puma to induce cell death, it was proposed that p53 activates the expression of PUMA, which then serves to release cytoplasmic p53 from the inhibitory interaction with Bcl-xL (Chipuk et al. 2004). However, the mechanism in which PUMA acts by liberating p53 from Bcl-xL in a way that it can bind and activate Bax on the mitochondria is controversial (Callus et al. 2008). p53 has been shown to activate the transcription of many genes, including *BAX* (Riley et al. 2008). Apoptosis by ER stress can occur through several pathways. Altered calcium homeostasis might contribute to the translocation of the death effectors Bax and

Hofmann 1999). The adaptor molecule FADD also contains a death effector domain (DED) that allows the homotypic recruitment of apoptotic initiator caspases containing DED, such as procaspase-8 in mice and procaspase-8 and -10 in humans (Muzio et al. 1996). These homotypic interactions lead to the formation of an oligomeric death-inducing signalling complex (DISC; Kischkel et al. 1995; Peter et al. 2003). The DISC forms a kind of molecular platform in which the initiator caspase-8 is conformationally activated, leading to autoproteolysis and proteolytic activation of the downstream effector caspases, such as caspase-3 and caspase-7. The extrinsic pathway can cross-talk with the intrinsic pathway through caspase-8-mediated cleavage of Bid (a BH3-only member of the Bcl-2 family proteins; Luo et al. 1998; Yang et al. 1998), which then triggers the release of mitochondrial proteins (Festjens et al. 2004). Two types of Fas receptor-induced apoptotic signalling have been discovered (reviewed in Krysko et al. 2007). Type I cells are characterized by high levels of DISC formation and increased amounts of active caspase-8 (Fig. 1.1). In this case, activated caspase-8 directly leads to the activation of downstream effector caspases without the need for a mitochondrial amplification loop. In type II cells, there are lower levels of DISC formation and, thus, lower levels of active caspase-8 (Scaffidi et al. 1998). In this case, signalling is dependent on the mitochondrial amplification loop, which involves the caspase-8-mediated cleavage of Bid to generate truncated (t) Bid, which induces the release of several mitochondrial factors (e.g. cytochrome c) that activate the mitochondrial apoptotic pathway (Fig. 1.1; Korsmeyer et al. 2000). Type II-induced apoptosis is blocked by Bcl-2 over-expression, whereas type I is not.

### *1.2.2 Intrinsic Pathway of Caspase Activation*

The intrinsic signalling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce either positive or negative intracellular signals. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure to suppress death programs, thereby trig-

Bak from the ER to the mitochondria (Ron et al. 2007). Inositol-requiring protein-1 (IRE1)-mediated activation of Jun N-terminal kinase (JNK) might contribute to cell death by phosphorylating and inactivating the anti-apoptotic regulator Bcl-2. The pro-death proteins Bax and Bak can assistant in IRE1 activation. Protein kinase RNA (PKR)-like ER kinase (PERK)-mediated phosphorylation of eukaryotic translation factor-2α (eIF2α) can contribute to cell death by inhibiting the synthesis of pro-survival proteins such as the transcription factor CHOP, may repress Bcl-2 expression (Ron et al. 2007). ATF4, activating transcription factor-4; GADD34, growth arrest and DNA-damage-inducible protein-34; DIABLO, direct IAP binding protein with low PI; EndoG, endonuclease G; HtrA, high temperature requirement protein A; IAP, inhibitor of apoptosis proteins; SMAC, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis protein. This figure is adapted from Vousden (2005); Krysko et al. (2007); Meier et al. (2007); Ron et al. (2007).

gering apoptosis. Positive stimuli include DNA damage induced by irradiation, chemotherapeutics and ER stress. The intrinsic pathway (Fig. 1.1) is mediated by mitochondria (Wang 2001) and it is regulated by the Bcl-2 family of pro- and anti-apoptotic proteins. Bcl-2 was initially described in an acute B-cell leukemia cell line as a gene linked to the immunoglobulin heavy chain locus due to chromosomal translocation (Pegoraro et al. 1984). Pro-apoptotic Bcl-2 proteins function to permeabilize the mitochondrial outer-membrane, which is accompanied by release of several proteins from the mitochondrial intermembrane space into the cytoplasm in response to apoptotic stimuli (Wang 2001; Festjens et al. 2004; Saelens et al. 2004). It has also been shown that besides its role as transcription factor in the nucleus, p53 also possesses an extranuclear function in that it directly binds anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) and activates the pro-apoptotic multi-domain Bcl-2 proteins (Bax and Bak) and thus regulates mitochondrial outer-membrane permeabilization (Fig. 1.1; Chipuk et al. 2004; Chipuk et al. 2006). However, this is still a matter of ongoing debate (Callus et al. 2008). Some of the well-characterized proteins released from mitochondria include cytochrome c, SMAC, (second mitochondria-derived activator of caspases/DIABLO, a direct inhibitor of apoptosis [IAP]-binding protein with low pI), AIF (apoptosis-inducing factor), EndoG (endonuclease G) and OMI/HTRA2 (high-temperature-requirement protein A2; van Loo et al. 2002; Vande Walle et al. 2008). Probably the most important of these pro-apoptotic proteins is cytochrome c, which binds to and activates the protein Apaf-1 in the cytoplasm (Li et al. 1997). Cytochrome c, first described in 1930, is imported into the intermembrane space as an apoprotein and then converted to holocytochrome c by addition of a haem group by the enzyme cytochrome c haem lyase (Mayer et al. 1995). In viable cells, it functions in the respiratory chain as an electron carrier between complexes III and IV. However, under apoptotic conditions, membrane integrity is lost and cytochrome c leaks into the cytosol. Interestingly, we observed mitochondrial heterogeneity during apoptosis: in a model of granulosa explants cultured under serum-free conditions, a subset of respiring mitochondria retains cytochrome c function until the stage of chromatin condensation and nuclear fragmentation (D'Herde et al. 2000; Krysko et al. 2001). In agreement with this observation, it has been shown that cytochrome c is never complete released from the mitochondrial fraction of anti-Fas-induced apoptotic L929 cells (Denecker et al. 2001) and from mitochondria treated in vitro with tBid. These data indicate that dysfunction of a fraction of mitochondria can provoke activation of the apoptotic program while the other fraction of mitochondria, with canonically localized cytochrome c, continues cellular respiration and ATP production until the stage of chromatin condensation and fragmentation. However, by fluorescent labeling of cytochrome c with green fluorescent protein in situ (Goldstein et al. 2000) and tetracysteine-containing sequence (Goldstein et al. 2005) it was shown that all mitochondria within a single cell release all their cytochrome c within five minutes in several cell types (NCI-H1299 and HeLa treated either with UV or with actinomycin D and TNF plus cycloheximide). The authors suggested a model of a 'single step' release of cytochrome c. This difference in kinetics of the mitochondrial response to apoptosis induction can depend on

whether the mitochondria are organized in a continuous (Amchenkova et al. 1988; Rizzuto et al. 1998; De Giorgi et al. 2000) or discontinuous (Collins et al. 2002; Collins et al. 2003) network in a cell. The different responses of mitochondria of the same cell type could be also related to culture conditions affecting the status of the mitochondrial networks (Egner et al. 2002) or to the strength of the cell death stimulus. Cytochrome c released into the cytoplasm acquires a cell death function because it can bind Apaf-1. This binding induces a conformational change that allows Apaf-1 to bind to ATP/dATP and to form the apoptosome complex (Jiang et al. 2000). The apoptosome functions as a platform for the recruitment of caspase-9 through homotypic CARD-CARD interactions, inducing a conformational change and activation of caspase-9 (Li et al. 1997; Rodriguez et al. 1999; Saleh et al. 1999; Zou et al. 1999). Activated caspase-9 converges again on the proteolytic activation of downstream effector caspases. Autoproteolysis of caspase-9 forms a negative feedback loop resulting in the release of caspase-9 from the apoptosome platform and its inactivation (Twiddy et al. 2004; Twiddy et al. 2006). In addition, Lakhani et al. (2006), using caspase-3 and caspase-7 deficient embryonic fibroblasts, provided evidence that these downstream caspases may amplify Bax translocation to mitochondria as well as cytochrome c release in response to ultraviolet radiation. This indicates that caspases-3 and -7 may participate in a feedback amplification loop to promote release of mitochondrial cytochrome c.

### *1.2.3 Organelles Other than Mitochondria Involved in Initiation of Apoptosis*

Accumulating evidence points to other organelles, including the endoplasmic reticulum (ER), the Golgi apparatus and lysosomes, as major points of integration of pro-apoptotic and anti-apoptotic signalling or cellular damage sensing (Hicks et al. 2005; Malhotra et al. 2007).

#### 1.2.3.1 The Endoplasmic Reticulum

The ER is a multifunctional organelle that has two main functions: it acts as the main cellular $Ca^{2+}$ store and it controls synthesis, folding and post-translational modification of proteins and lipids (Groenendyk et al. 2005). The ER participates in initiating apoptosis by at least two different mechanisms, namely, $Ca^{2+}$ signalling and the unfolded protein response. In support of the first mechanism is that the Bcl-2 family members localize not only in mitochondria but also in the ER. Thus, Bcl-2 proteins interrupt calcium homeostasis and lead to $Ca^{2+}$ release from the ER, modulating cell survival and cell death signals. In addition prolonged ER stress can be responsible for activation of apoptosis (Nakamura et al. 2000; Groenendyk et al. 2005; Bernardi et al. 2007). Identification of caspase-12 on the cytoplasmic side of the ER and demonstration

that caspase-12 is processed in cells treated with ER-stress agents favored the idea that it might be the initiator caspase in ER-stress-mediated apoptosis (Nakagawa et al. 2000). However, later on it was discovered that although caspase-12 is processed in ER-stress-mediated cell death in B16/B16 melanoma cells, the cells die to the same extent in the absence of caspase-12 (Kalai et al. 2003). Similarly, it was shown that murine cells lacking caspase-12 expression were not protected from apoptosis induced by ER stress agents (Obeng et al. 2005; Di Sano et al. 2006). Besides, over-expression of the anti-apoptotic Bcl-$x_L$ could provide protection for cells from ER stress-induced death (Obeng et al. 2005). These data suggest that although caspase-12 is processed in apoptosis mediated by ER stress, it may be dispensable for the execution of cell death prompted by ER stress (Lamkanfi et al. 2004). In addition, the existence of truncated human caspase-12 or enzymatically inactive caspase-12 (Lamkanfi et al. 2004) makes it unlikely that it would play a major role in neurodegenerative diseases such as Alzheimer's disease, a role that has been suggested on the basis of the reduced cytotoxicity of the β amyloid peptide in caspase-12-deficient mice (Nakagawa et al. 2000). In this regard, caspase-12 seems to be the cFLIP counterpart for regulating the inflammatory branch of the caspase cascade. In mice, caspase-12 deficiency confers resistance to sepsis and its presence exerts a dominant-negative suppressive effect on caspase-1, resulting in enhanced vulnerability to bacterial infection and septic mortality (Saleh et al. 2006). Several other ER-associated pro-apoptotic molecules have been reported, such as Bap31, a polytopic integral protein of the ER membrane that can bind caspase-8 and Scotin, which are involved in p53-mediated apoptosis (Lamkanfi et al. 2004). Other mechanisms the protein kinase RNA (PKR)-like ER kinase (PERK) / eukaryotic translation initiation factor-2α (eIF2α)—dependent transcription induction of the pro-apoptotic transcription factor CHOP; Bak/Bax-regulated $Ca^{2+}$ release from the ER; inositol requiring protein 1 (IRE1)—mediated activation of apoptosis signal-regulating kinase 1 (ASK1) / Jun N-terminal kinase (JNK; Fig. 1.1; reviewed in detail by Malhotra et al. 2007; Ron et al. 2007).

#### 1.2.3.2 The Golgi Apparatus

The Golgi apparatus consists of a series of parallel cisternae and vesicles that carry molecular ''cargo''. Its major function is to mediate protein and lipid modification, transport and storage. Recent studies suggest that the Golgi complex can also sense and transduce apoptotic signals. The discovery of a pool of caspase-2 at the cytoplasmic face of the Golgi complex indicates that caspase-2 may play a key role in apoptotic signalling at the Golgi complex. During apoptosis, the Golgi apparatus is disassembled due to caspase-mediated cleavage of golgins. These are proteins that maintain the structural and functional integrity of this organelle. Golgin-160 can be cleaved by caspase-2, -3, and -7. The cleavage of golgin-160 by caspase-2 occurs rapidly and precedes caspase-3 cleavage, indicating an early role in apoptosis for caspase-2 activation at the Golgi complex (Mancini et al. 2000). Furthermore, HeLa

cells expressing a caspase-resistant mutant of golgin-160 were resistant to apoptosis induced by ligation of death receptors and by drugs that induce ER stress, but they were sensitive to other pro-apoptotic stimuli, including staurosporine, anisomycin, and etoposide (Maag et al. 2005). Several studies also indicate that another Golgi protein, namely p115, is also cleaved early in the apoptosis (Chiu et al. 2002; Mukherjee et al. 2007). Moreover, because the cytoskeleton maintains organization of the Golgi apparatus, it was also suggested to be involved either in Golgi apparatus disassembly or in disruption of the cytoskeleton, which could lead to onset of apoptosis. Mukherjee et al. (2007) showed that neither actin nor alpha-tubulin was cleaved in Fas-mediated apoptosis, which indicates that Golgi fragmentation precedes breakdown of the cytoskeleton. Taken together, these data indicate that some apoptotic signals may be sensed and integrated at the Golgi membranes. The Golgi complex may provide a link between ligation of death receptors and the ER stress response.

#### 1.2.3.3 The Lysosomes

Lysosomes, which have been given the epithet 'suicide bags' (De Duve, Nobel prize speech, 1974), consist of numerous acid vesicles that contain many catabolic hydrolases received from the Golgi network. Their substrates are from inside the cell (autophagy) as well as from outside (heterophagy). These hydrolases contribute to type II (autophagic) cell death (discussed later). About 15 years ago, lysosomal membrane destabilization was for the first time considered as an early event in apoptosis. However, a recent study confirmed that lysosomal rupture may be an upstream event in some forms of apoptosis (Kurz et al. 2008). Although cathepsins may function at a late stage of apoptosis (Foghsgaard et al. 2001), they were also reported to translocate from lysosomes to the cytosol during early apoptosis, before cytochrome c release and caspase activation. Additionally, cathepsin-mediated activation of Bax and Bak indicates that cathepsins may induce apoptosis by the mitochondrial pathway (Deiss et al. 1996; Ferri et al. 2001; Blomgran et al. 2007). In conclusion, each organelle may possess sensors that detect specific alterations, locally activate signal transduction pathways, and emit signals that ensure inter-organelle cross-talk (Ferri et al. 2001).

Apoptosis is considered as a carefully regulated energy-dependent process, characterized by specific morphological and biochemical features in which caspase activation plays a central role. The importance of understanding the mechanistic machinery of apoptosis is vital because apoptotic cell death is a component of both health and disease, being initiated by various physiological and pathological stimuli. Disturbance of cell death regulation can be an important component of diseases, such as cancer, autoimmune lymphoproliferative syndrome, AIDS, ischemia, and neuron-generative diseases, e.g., Parkinson's disease, Alzheimer's diseases, Huntington's disease, and Amyotrophic Lateral Sclerosis. Some of these examples are discussed in more detail in Diez-Fraile et al. (Chap. 2, this Vol.). Notably, several human diseases can arise when apoptotic cells are not cleared

sufficiently by phagocytes. In the second part of this book, the possible contribution of disturbed clearance of apoptotic cells to the pathogenesis of the following human diseases are discussed: systemic lupus erythematosus (Mevorach, Chap. 10, this Vol.), chronic lung diseases (cystic fibrosis, non-CF bronchiectasis, chronic obstructive pulmonary disease) and asthma (Reynolds and Hodge, Chap. 14, this Vol.), atherosclerosis (Martinet et al., Chap. 13, this Vol.), and cancer (Bartunkova and Spisek, Chap. 12, this Vol.). The potential for using apoptotic cells to treat or prevent graft rejection and autoimmune disorders is discussed by Divito and Morelli (Chap. 11, this Vol.).

## 1.3 Autophagy

In normal cells, two general mechanisms are involved in the degradation and recycling of the building blocks of organelles, proteins and other components of the cytoplasm. In the large-scale degradation of components of the cytoplasm, short-lived regulatory proteins are broken down to amino acids by the ubiquitin-proteasome system, and long-lived structures and proteins are targeted to the lysosome for hydrolysis by autophagy. Autophagy can be described as a process of cell recycling through degradation by lysosomes. It involves intracellular membrane reorganization to create auto-phagosomes, which sequester cytoplasm and organelles. After that they fuse with lysosomes and the cargo is degraded and recycled (Kelekar 2005; Hoyer-Hansen et al. 2008).

Several forms of autophagy have been described (Baehrecke 2005), including microautophagy and macroautophagy (hereafter referred to as autophagy) because of its association with type II cell death. In brief, several steps during autophagy can be distinguished: initiation, cargo packaging, maturation (docking and fusion) and breakdown (Fig. 1.2). First, a phagophore is generated; this is an isolated membrane that sequesters cytoplasm and organelles, or organelle fragments. The phagophore then expands to form an autophagosome, a double-membrane structure. During maturation, auto-phagosomes deliver their cargo to lysosomes by fusing with them to form auto-phagolysosomes. This compartment contains a range of hydrolases that can degrade proteins, lipids, nucleic acids and carbohydrates, which may lead to organelle degradation (Klionsky et al. 2000; Kelekar 2005; Lleo et al. 2007). Several origins have been proposed for the wrapping membrane structure. Among these are the ribosome-free regions of the rough endoplasmic reticulum and the *trans* Golgi network, but it is now believed that the autophagosome is formed mostly *de novo* from core membrane that expands through vesicular addition (Petiot et al. 2002; Klionsky 2007). Since the first description of autophagy (De Duve 1966, Ph.D. thesis), numerous studies have described it as a survival mechanism under poor nutritional conditions or birth-related starvation. Molecular mechanisms (Fig. 1.2) implicated in regulating autophagy in response to starvation were initially discovered in *Saccharomyces cerevisia*. Screens for yeast mutants that were star-

vation-sensitive or defective in the degradation of specific cytosolic proteins produced different mutants: *Apg* (autophagy-defective), *Aut* (autophagocytosis), and *Cvt* (cytoplasm-to-vacuole targeting) mutants; these partially overlap and are collectively designated as *Atg* genes (Tsukada et al. 1993; Thumm et al. 1994; Harding et al. 1995; Baba et al. 1997).

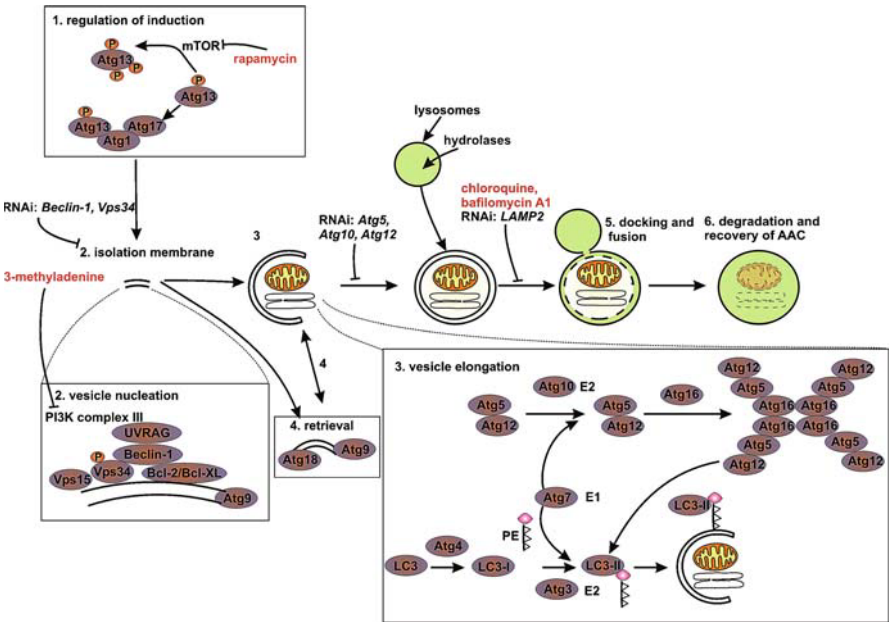


**Fig. 1.2** Molecular mechanisms of autophagy. A portion of cytoplasm, including organelles, is enclosed by an expanding membrane sac, the phagophore (also called isolation membrane). Step first involves the de-repression of the mTOR Ser/Thr kinase, which inhibits autophagy by phosphorylating Atg13. This leads to the dissociation of Atg13 from a protein complex that contains Atg1 kinase and Atg17, and thus attenuates the Atg1 kinase activity. When mTOR is inhibited, re-association of de-phosphorylated Atg13 with Atg1 stimulates its catalytic activity and induces autophagy. Step two is vesicle nucleation starts from the activation of mammalian Vps34, a class III phosphatidylinositol 3-kinase (PI3K), to generate phosphatidylinositol-3-phosphate. Vps32 activation depends on the formation of a multiprotein complex in which Beclin-1, UVRAG and a myristylated kinase (Vps15) participate. The third step is auto-phagosomes formation (vesicle elongation) and involves two pathways: conjugation of Atg12 to Atg5, with the help of the E1-like enzyme Atg7 and the E2-like enzyme Atg10; conjugation of phosphatidylethanolamine (PE) to LC3 by the sequential action of the protease Atg4, the E1-like enzyme Atg7 and the E2-like enzyme Atg3. This leads to the conversion of the soluble form of LC3-I to the autophagic-vesicle-associated form LC3-II. The outer membrane of the auto-phagosomes subsequently fuses with a lysosome (forming autolysosomes), exposing the inner single membrane of the autophagosome to lysosomal hydrolases (step 5, docking and fusion). Finally the cargo-containing membrane compartment is lysed, and the contents are degraded or aminoacids (ACC) recovered and used for nutrients (step 6, degradation and recover of AAC). Chemical compounds are shown in red. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme. This figure is adapted from Maiuri et al. (2007); Levine et al. (2008).

### 1.3.1 Autophagy as a Strategy for Cell Survival

The pro-survival function of autophagy is an ancient process conserved from yeast to mammals. In response to starvation, *C. elegans* larvae enter dauer, a latent developmental state, and RNAi-inactivation of autophagy genes (*bec-1*, *atg8* and *atg18*) disrupts normal dauer formation (Melendez et al. 2003). A recent study demonstrated that autophagic degradation and recovery of biomolecules within early embryos is essential for preimplantation in mammals (Tsukamoto et al. 2008) pointing to autophagy as a unique strategy for supporting mammalian development. These results are not inconsistent with the finding that conventional $Atg5^{-/-}$ mice survive preimplantation development (Kuma et al. 2004). However, these $Atg5^{-/-}$ mice were generated by mating $Atg5^{+/-}$ mice, so maternally inherited Atg5 protein in the cytoplasm of Atg5-null oocytes might have rescued the autophagy-deficient phenotype during early embryogenesis (Kuma et al. 2004; Tsukamoto et al. 2008). Rather, these two different mouse models clearly demonstrate the specific importance of autophagy as a survival mechanism during very early post-fertilization development, in which maternally inherited Atg5 protein remains in the cytoplasm. Autophagy genes may also be critical for maintaining cellular survival when cells are unable to take up external nutrients, e.g., during growth factor deprivation. Growth factor withdrawal usually results in rapid apoptotic cell death, but recent studies in apoptotic-deficient $bax^{-/-}$, $bak^{-/-}$ cells raveled an important role for autophagy genes in maintaining cellular survival following IL-3 deprivation (Lum et al. 2005). IL-3-deprived $bax^{-/-}$, $bak^{-/-}$ cells activate autophagy and ultimately die. Notably, the addition of growth factor anytime before death reverses the catabolic process and keeps the cells alive (Lum et al. 2005). Furthermore, RNAi against *beclin1*, *atg5*, *atg10*, and *atg12* enhances starvation-induced, but not staurosporine-induced, apoptotic cell death. This finding illustrates an interesting mechanism by which autophagy genes promote survival during nutrient deprivation, namely, suppression of the apoptotic death pathway (Boya et al. 2005). Autophagy occurs in almost all cells to help maintain homeostasis. It is also activated when cells need to generate energy during starvation or growth factor deficiency (Levine et al. 2008). Together, these data suggest that autophagy is a critical survival mechanism to overcome stress conditions.

### 1.3.2 Cell Death Associated with Autophagy

Paradoxically, the presence of autophagic structures in dying cells may also indicate the existence of type II autophagic cell death (Schweichel et al. 1973). Type II or autophagic cell death is a type of cell death accompanied by extensive autophagic vacuolization of the cytoplasm (Okada et al. 2004; Kroemer et al. 2005). In autophagic cell death, partial chromatin condensation occurs late if at all, and no DNA laddering is observed (Kroemer et al. 2005; Levine et al. 2005). It is important

to emphasize that an optimal identification of autophagic cell death would require the use of transmission electron microscopy to distinguish auto-phagosomes from other types of vesicles such as endosomes, lysosomes and macropinosomes. Cell death associated with autophagy in most instances was found during the destruction of organs and large cell units in mouse and rat embryos and fetuses (Schweichel et al. 1973). Another example may be insect metamorphosis, in which cell death is typically autophagic, and blocking autophagy before pupation and die during metamorphosis (Juhasz et al. 2003). Autophagic cell death also has been observed in adult insects and vertebrates, including humans; it is often associated with the elimination of large secretory cells during the adjustment of sexual organs and ancillary tissues to seasonal reproduction (Bursch et al. 2000).

However, it is important to stress that whether type II cell death is a separate form of cell death remains unclear (Lockshin et al. 2004). Autophagosome formation during cell death does not necessarily means autophagic cell death. Pure autophagic cell death should be defined by the presence of auto-phagosomes simultaneously with the absence of apoptotic and necrotic hallmarks. Moreover, induction of autophagy (by starvation or rapamycin) should result in an increase in cell death and suppression of autophagy should lead to cell survival (White 2008). Yet, the literature contains strong evidence to support the notion that autophagic cell death may overlap either with caspase-dependent or caspase-independent cell death. Studies of steroid-triggered death of salivary gland cells during Drosophila development showed that caspases function in autophagic death of cells. Caspase-3 activation and *atg* gene transcription immediately precede autophagic cell death in salivary glands (Lee et al. 2001; Lee et al. 2003; Martin et al. 2004). Similarly, it has been shown that the pro-apoptotic signalling molecule, TNF-related apoptosis-inducing ligand (TRAIL), regulates autophagy in an in vitro model of mammary gland formation (Mills et al. 2004). In that study, the increased level of TRAIL was detected during morphogenesis of MCF-10A mammary epithelial cells in 3D basement-membrane cultures just before the death of luminal cells expressing active caspase-3, whereas these cells possess autophagic cell death morphology. Additionally, when lysosome-associated membrane protein-2 (LAMP2) was inactivated by RNAi or by homologous recombination in cells cultured in nutrient-deficient conditions, the cells accumulated autophagic vacuoles and then died with hallmarks of apoptosis, namely, caspase activation and chromatin condensation (Gonzalez-Polo et al. 2005). Other molecular links between autophagy and apoptosis may be the death-associated protein kinase (DAPk) and DAPk-related protein 1, which regulate membrane blebbing during apoptosis, but can also promote autophagic vacuole formation in dying cells (Inbal et al. 2002). It has been shown that Bcl-2 binds to Beclin 1 and disrupts its autophagic function, and that in the absence of Bcl-2 binding, *Beclin 1* mutants induce excessive autophagy and promote cell death (Pattingre et al. 2005). This study demonstrates the Beclin 1-Bcl-2 complex may function as a rheostat that ensures that autophagy levels remain within a physiological range rather than in a non-physiological range that triggers cell death (see Fig. 1.2 for an overview of molecular pathways). In addition, it has been demonstrated that Bcl-2 phosphorylation by the stress-activated signalling molecule, JNK1, is required

for its starvation-induced dissociation from Beclin 1 and subsequent activation of autophagy (Wei et al. 2008). Taken together, these studies indicate that complex interrelationships may exist between autophagy and apoptotic cell death pathways.

In spite of the accumulating evidence for overlap of autophagic cell death with apoptosis, autophagy may also be related to caspase-independent cell death. It was shown that cell death induced by the human homologue of the Drosophila spin gene product (HSpin1; Gonzalez-Polo et al. 2005) is caspase-independent and autophagic (Yanagisawa et al. 2003). In support of this idea, RNAi directed against two autophagy genes, *atg7* and *beclin1*, severely affected cell death in mouse L929 cells treated with the caspase inhibitor zVAD-fmk (Yu et al. 2004). In this model, a new molecular pathway was defined: activation of Rip1 (a serine-threonine kinase) and Jun amino-terminal kinase induced cell death with the morphology of autophagy. Inhibition of caspase-8 induces cell death with autophagic morphology (Yu et al. 2004). Similarly, the autophagic cell death of *Bax*$^{-/-}$*Bak*$^{-/-}$ mouse embryonic fibroblasts treated with either etoposide or staurosporine was prevented by the knock-down of *Beclin1* and *Atg5* (Shimizu et al. 2004), suggesting that autophagic cell death occurs in a caspase-independent manner. In addition, these data might be interpreted to mean that either apoptosis effectors, such as caspases, Bax and Bak, actively suppress the autophagic components of cell death, or that the autophagic and apoptotic effector mechanisms constitute backup mechanisms that come into action when one or the other lethal pathway is inhibited (reviewed in Krysko et al. 2007).

It is now clear that autophagy has multiple roles. On one hand, autophagy is a mechanism for degrading long-lived proteins and damaged organelles through the auto-phagolysosomal pathway. On the other hand, it serves as a survival pathway preventing or delaying cell death. This cell survival mechanism is activated in cells undergoing different forms of cellular stress. On the other hand, when stress continues, autophagy may eventually become a cell death mechanism. Despite recent advances in the understanding of molecular mechanisms and biological functions of autophagic cell death, it is not clear whether it is an alternative way of dying that differs from apoptotic and necrotic cell death, or whether failure of autophagy to rescue the cell can lead to cell death by either pathway. It is also conceivable that the extent of autophagy determines the decision to survive or to die.

### *1.3.3 Autophagy and Clearance of Dying Cells*

It was recently shown that cells dying by autophagy or by another mechanism associated with autophagy are engulfed by professional and non-professional phagocytes. Phosphatidylserine (PS) exposure mediates recognition and engulfment of cells dying by autophagy (Petrovski et al. 2007a). Phagocytic uptake of dying autophagic cells by macrophages leads to a pro-inflammatory response characterized by the production of IL-6, TNF and IL-8 (Petrovski et al. 2007b). However, there is only limited knowledge of the molecular mechanisms of recognition and

phagocytic uptake of cells dying in association with autophagy and of its functional consequences; additional in depth studies are required.

Several studies indicate that autophagy may be involved in optimal clearance of apoptotic cells. Recently, data was obtained on embryonic cavitation, which is the earliest programmed cell death process in mammalian development. It was shown that inner ectodermal cells in embryonic bodies lacking the essential autophagic gene *Beclin1* or *Atg5* underwent apoptosis normally but were not engulfed by neighboring cells, because they fail to express PS and because they secrete lower levels of lysophosphatidylcholine (LPC; Qu et al. 2007). However, it is important to note that this phenomenon was observed for Beclin1$^{-/-}$ embryos in vitro and in vivo, while for Atg5$^{-/-}$ embryos only in vitro data were obtained. Further studies will resolve this contradiction. Another report showed that treatment of retinas with the autophagic inhibitor 3-methyladenine resulted in accumulation of numerous apoptotic cells (Mellen et al. 2008). This was accompanied by the absence of the 'eat me' PS signal and by deficiency of subsequent clearance of apoptotically dying cells. These studies demonstrated that autophagy, beyond its survival and cell death function, might also be essential in the exposure of 'eat-me' signals on apoptotic cells, which indicates that autophagosome formation during apoptosis may be one of the mechanisms used to expose phagocytosis signals on the surface of dying cells.

### *1.3.4 Role of Autophagy in Human Diseases*

Autophagy is a highly conserved process enabling clearance of excess or aberrant organelles and long-lived proteins (discussed above). This process also plays important roles in embryogenesis, tissue remodelling, degradation of intracellular pathogens (Yorimitsu et al. 2005), and adaptation to changing environment conditions. Importantly, autophagy is also involved in both preventing and contributing to some types of human diseases (Yorimitsu et al. 2005; Levine et al. 2008). Several human pathologies are associated with increased auto-phagosomes formation, such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, transmissible spongiform encephalopathies, and Huntington's disease). Recent studies indicate that activation of autophagy and accumulation of auto-phagosomes in patients' brains play a protective role because they enable clearance of aggregates of mutant proteins that are characteristic of neurodegenerative diseases (Kelekar 2005; Levine et al. 2008). For example, in Huntington's disease is found a mutated huntingtin protein (mHtt) with an abnormal number of glutamine residues (polyQ), mutant alpha-synucleins were observed in Parkinson's disease, and different mutations of tau proteins were found in temporal dementia (Williams et al. 2006). These potentially toxic oligomeric and aggregated proteins are inaccessible to proteasomes and so they are degraded through autophagy (Levine et al. 2008). Similarly, extensive accumulation of auto-phagosomes in the muscle and leading to cardiomyopathy is characteristic of a Danon disease. In this case, myopathy is a result of the lysosomes' inability to fuse with auto-phagosomes due to a mutation in LAMP-2 protein, which is involved in maturation of auto-phagosomes. Other myopathies associated

with autophagic vacuole accumulation are X-linked myopathy and inclusion body myositis (Kelekar 2005).

In several experimental models it has been shown that autophagy could function as a back up mechanism when cells fail to execute apoptosis (Yu et al. 2004; Levine et al. 2005; Lum et al. 2005; Lefranc et al. 2007). It is well known that cancer cells are characterized by deregulated cell proliferation and suppression of apoptosis. Autophagy activation can have contradictory consequences for tumour development. On one hand, recent studies indicate that autophagy is important for tumour progression (Hippert et al. 2006). Since autophagy is known to be a survival mechanism that prevents cells from dying in stressful conditions (discussed above) it could also serve as a survival mechanism in solid tumours, where limited angiogenesis leads to nutrient depravation and reduced oxygen levels (Hippert et al. 2006; Hoyer-Hansen et al. 2008). On the other hand, it has been proposed that autophagy has an anti-cancer role as well. Strong evidence connecting autophagy with cancer appeared in the late 1990s, when it was discovered that the ATG gene *Beclin-1* may serve as a tumour suppressor (Qu et al. 2003; Klionsky 2007). It was proposed that autophagy can inhibit tumour development in the early stage. Later, other potential tumour suppressor genes were identified, including UV irradiation resistance-associated gene *(UVRAG)*, *Atg4C*, and damage-regulated autophagy modulator *(DRAM*; Hoyer-Hansen et al. 2008). Interestingly, p53 positively regulates autophagy, thereby corroborating the notion of tumour suppression by autophagy. In this regard, genotoxic stress caused by DNA-damaging agents induces p53-dependent autophagy (Feng et al. 2005; Zeng et al. 2007). Likewise, oncogenic activation, stimulated by forced expression of ARF or p53, induces autophagy in human cancer cells (Abida et al. 2008). Surprisingly, inhibition of p53 with pfithrin-α, knockdown of *p53* with siRNA, and genetic deletion of *p53* increases autophagy, whereas basal levels of p53 apparently inhibit autophagy (Tasdemir et al. 2008). The study by Tasdemir et al. (2008) revealed further complexity in the homeostatic regulation by showing that p53 and autophagy are interconnected in a hitherto unexpected bi-directional fashion. Although how autophagy inhibits tumour progression is still unclear, several hypotheses have been proposed to explain how this pro-survival mechanism can contribute to inhibition of cancer development. One hypothesis is that autophagy may suppress tumourigenesis by reducing necrotic cell lysis and therefore limiting tumour inflammation (Hoyer-Hansen et al. 2008). Another hypothesis is that autophagy suppresses tumour development by removing damaged mitochondria, thus reducing production of ROS, which is a potential source of DNA damage (Levine 2006; Mathew et al. 2007). Yet another hypothesis is that autophagy may influence cell cycle by degrading proteins responsible for regulation of cell growth and thereby leading to slower proliferation of tumour cell lines (Koneri et al. 2007; Levine et al. 2008). Taken together, these data indicate that autophagy may both stimulate and inhibit cancer, depending on the context.

The occurrence of autophagy in cancer cells may provide the grounds for using inhibitors or inducers of autophagy in the treatment of cancer. Autophagy can be pharmacologically inhibited by bafilomycin A1 (which interferes with phagosome-lysosome fusion) or by targeting 3-methyladenine to class III PI3K, which is

involved in autophagosome formation (Levine et al. 2008). However, the autophagy inhibitors that are clinically relevant are rather few at the moment. These include microtubule-disrupting agents (vincristine and paclitaxel), which are used for treatment of various forms of cancer (Groth-Pedersen et al. 2007), and chloroquine, which is in a pre-clinical trial as a sensitizer to radiotherapy and chemotherapy (Amaravadi et al. 2007; Hoyer-Hansen et al. 2008). Inducers of autophagy include rapamycin, xestospongin B and lithium (Levine et al. 2008). Importantly, several trials have been started to examine the potential usefulness of mTOR inhibitors (Fig. 1.2; rampamycin, CCl-779), EB1089 (targets a vitamin D receptor) and angiogenesis inhibitors (anti-VEGF anibody, ADH-1, Kringle5) in treatment of numerous cancers, such as breast, prostate, brain, lung, ovary and cervix carcinomas (Noda et al. 1998; Ambalavanan et al. 2005; Nguyen et al. 2007). Moreover, combining autophagy inhibitors with other metabolic inhibitors may result in a synergic effect. Cancer cell death was reported when the lysosome-damaging drug siramesine was combined with blockade of autophagy by removal of Atg proteins or treatment with microtubule destabilizing drugs (vincristine, taxotere or 2-methoxyestradiol) (Escuin et al. 2005). On the other hand, some alkylating chemo-therapeutic agents (actinomycin D), gene therapy (p53), cytokines and radiation have been shown to induce cell death type II in various cancer cell lines (Lefranc et al. 2007; Hoyer-Hansen et al. 2008), but their potential effect still needs to be verified in vivo.

In conclusion, targeting autophagy may represent an alternative way for treatment of different diseases, including tumours resistant to radiotherapy and pro-apoptotic chemotherapy. Yet, the uses of pro- or anti-autophagic treatment still needs to be validated in vivo. Understanding autophagy may ultimately allow scientists and clinicians to harness this process for the purpose of improving human health.

## 1.4 Necrosis

Type III cell death, according to the classification of Schweichel and Merker, is characterized by swelling of the endoplasmic reticulum, mitochondria, and the cytoplasm, with subsequent collapse of the plasma membrane and lysis of the cells (Schweichel et al. 1973). The term oncosis was proposed to refer to the early stage of primary necrosis, during which cells committed to death pass through a pre-lethal process in which they swell (Majno et al. 1995). Notably, in the absence of phagocytosis, apoptotic cells may lose their cytoplasmic membrane integrity and proceed to a stage called secondary necrosis. Secondary necrotic cells resemble necrotic cells, but they have already gone through an apoptotic stage that can still be recognized at the ultrastructural level by the typical chromatin condensation pattern (reviewed in Krysko et al. 2007). It is important to distinguish necrosis from other forms of cell death, particularly because it is often associated with unwarranted loss of cells in human pathologies. However, there is no clear biochemical definition of necrotic cell death and consequently no positive biochemical markers that unambiguously discriminate necrosis from apoptosis. Therefore, we propose that the definition of

cell death types should be based on three different criteria: morphological features, biochemical features, and interactions with phagocytes (Krysko et al. 2008).

Necrotic cell death was often considered to be a passive process, lacking underlying signalling events and occurring under extreme physico-chemical conditions, such as abrupt anoxia, sudden shortage of nutrients, heat and detergents. However, in disagreement with the classical textbook notion that it is merely an accidental consequence of non-physiological stress, necrosis might actually be programmed in terms of both its course and its occurrence. This idea is supported by several data. In several physiological and pathological conditions, necrosis is described as a form of caspase-independent cell death. For example, necrosis has been found to be a potential substitute for apoptosis during development. The loss of interdigital cells in the mouse embryo, a prototype of programmed cell death, still occurs by necrosis either upon inhibition of caspases by drugs, or in mice bearing a mutation in the *apaf-1* gene (Chautan et al. 1999). Caspase-independent cell death is also involved in processes such as the negative selection of lymphocytes (Smith et al. 1996; Doerfler et al. 2000; Jaattela et al. 2003), TNF-mediated liver injury (Kunstle et al. 1999) and the death of chondrocytes controlling the longitudinal growth of bones (Roach et al. 2000). Necrotic cell death is also instrumental during cellular turnover in the human large intestine (Barkla et al. 1999) and in response to viral infection (Chan et al. 2003). Susceptibility to necrotic death can be regulated by genetic and epigenetic factors. It has been shown that mouse strains vary in their susceptibility to brain ischemia, which was also dependent on the age of the mice and the brain regions (Mattson et al. 2006). In addition, necrotic cell death is involved in the pathogenesis of several human pathologies, such as neurodegeneration, ischemia-reperfusion and infection (reviewed in Vanlangenakker et al. 2008).

Next, we would like to consider some biochemical mechanisms implicated in programmed necrotic cell death. Depending on the cell line used, TNF can induce either apoptotic or necrotic cell death (Laster et al. 1988). In fibrosarcoma L929 cell line, TNF induces necrotic cell death without the involvement of caspases, even though stimulation of Fas leads to classical apoptosis in the same cells (Vercammen et al. 1998b). The necrotic pathway triggered in the L929 cell line by FasL can be detected only before the inactivation of the caspases by zVAD-fmk (Vercammen et al. 1998a). Thus, depending on the cellular context, both death domain-containing receptors (TNFR1 and Fas) can initiate apoptotic or necrotic cell death. This death receptor-induced caspase-independent pathway is not restricted to the L929 cell line. Similarly, in the U937 or Jurkat T lymphocyte cell lines, TNF, Fas and TRAIL trigger necrotic cell death when caspases are inhibited (Khwaja et al. 1999; Matsumura et al. 2000; Holler et al. 2000). Besides that, TNF in the presence of caspase inhibitors can induce caspase-independent cell death in murine embryonic fibroblasts (MEFs; Bernardi et al. 2006). FADD is an important adaptor molecule serving as a platform for the initiation of apoptotic as well as necrotic cell death. Over-expression of a FADD containing only DD (FADD-DD) leads to necrosis, while over-expression of FADD containing only DED (FADD-DED) kills the L929 cells by apoptosis; also, cell death can be redirected from apoptosis to necrosis in the presence of zVAD-fmk (Boone et al. 2000; Vanden Berghe et al. 2004). These

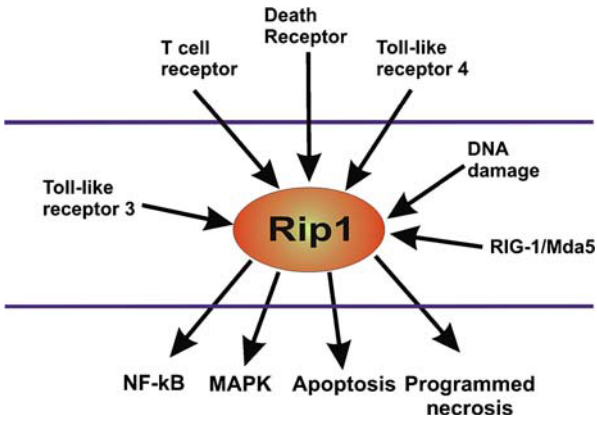


**Fig. 1.3** Rip1 is a bifurcation point between cell death and cell live. Several signalling pathways converge on Rip1 such as activation of the T cell receptors, death receptors and TLR-3 and -4, and signalling pathways initiated upon detection of intracellular stress (dsRNA or DNA damage). The activation of NF-κB, MAPKs, apoptosis or necrosis is dependent on the cellular context. This figure is adapted from Festjens et al. (2007). dsRNA, double stranded RNA; MAPK, mitogen activated protein kinase; Rig1, retinoic acid inducible gene-1; Mda5, melanoma differentiation-associated gene 5.

data suggest that a bifurcation between apoptosis and necrosis might be situated at the level of the adaptor protein FADD. Binding of caspase-8 to DED of FADD leads to its activation and subsequent apoptosis, but if the recruitment or the enzymatic activity of caspase-8 is prevented, the presence of death domain of FADD in the receptor complex leads to necrosis (Boone et al. 2000; Vanden Berghe et al. 2004). Another molecule involved in the necrotic signalling cascade is Rip1 (Fig. 1.3). A study employing FADD- and Rip1-deficient Jurkat cells identified the kinase Rip1 as a crucial component of Fas- and TNF-mediated necrotic cell death (Holler et al. 2000). TNF signal is transduced by homotypic interaction of DD with RIP1, which allows Rip1 to bind directly to the TNFR1 or indirectly through TRADD (Stanger et al. 1995; Harper et al. 2003). It is interesting to note that Rip1, a key mediator in the necrotic cell death, can be cleaved by activated caspase-8 (Lin et al. 1999; Martinon et al. 2000), indicating that apoptotic and necrotic cell death pathways interfere with each other, and that the initiation of apoptosis might actively suppress the necrotic pathway. In addition, studies on the heat shock protein (Hsp) 90 (Kalai et al. 2002) have also revealed the importance of Rip1 in necrotic signalling. Necrosis induced by Fas or TNFR1 is inhibited by the Hsp90 inhibitors geldanamycin and radicicol, which are responsible for a strong down-regulation of Rip1 levels (Lesnefsky et al. 2001; Ma et al. 2005).

Degterev et al. (2005) characterized “necrostatins” as the first class inhibitors of necrotic cell death. To distinguish programmed necrosis that occurs in the absence of caspase activation from accidental necrosis, these authors named it “necroptosis”. Later on the same group demonstrated that necrostatins inhibit Rip1 kinase activity, the key upstream kinase involved in the activation of programmed necro-

sis (Degterev et al. 2008). This discovery has a great potential in the treatment of human pathologies, such as myocardial infarction and cerebral ischemia, in which cell death exhibits necrotic features. Consequently, necrostatins have recently been shown to reduce histopathology and improve functional outcome after controlled cortical impact in mice (You et al. 2008). The mechanism that leads to the execution of necroptosis downstream of Rip1 kinase activation remains unclear and the subject of active research, especially the possible role of Rip1 ubiquitination and deubiquitination in this respect could be influence the biological outcome of Rip1 between apoptosis, necrosis or NF-κB activation (Bertrand et al. 2008). Ample evidence suggests that excessive formation of reactive oxygen species (ROS) is involved. Inhibition of caspases (which sensitizes to necrosis) results in increased formation of ROS. The addition of the scavenger butylated hydroxyanisole (BHA) protects L929 cells from TNF-induced necrosis, but not from Fas-mediated apoptosis (Vercammen et al. 1998b; Goossens et al. 1999), while necrosis induced by Fas is also blocked by BHA (Vercammen et al. 1998a). The strong protective effect of BHA may go beyond its oxygen radical scavenging activity. Festjens et al. (2006a) reported that BHA may directly inhibit several mechanisms that are implicated in necrotic cell death, such as complex I activity, PLA2 activation and lipoxygenase activity.

A connection between Rip1 and a non-mitochondrial source of ROS is the plasma membrane-associated NADPH oxidase-1 (Nox1). Knockdown of Nox1, which is responsible for TNF-induced generation of superoxide anions, delays necrosis in mouse fibroblasts. In these cells, TNF treatment induces Nox1 activation through a Rip1-dependent signalling complex containing TRADD, NOXO1 and the small GTPase Rac1 (Kim et al. 2007; Vanden Berghe et al. 2007).

Besides death receptor-induced necrosis, triggering pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs), the cytosolic NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) leads to initiation of inflammation and/or cell death (Fig. 1.3; Vanlangenakker et al. 2008). Synthetic dsRNA, via TLR3, induces necrotic cell death in human Jurkat cells and murine L929 fibrosarcoma cells in a caspase-8 and FADD-independent manner, and type I and II-interferons (IFNs) can sensitize for this necrosis (Dennis et al. 1991). Also, the gram-negative bacterium *Shigella flexneri* induces necrosis in neutrophils by activating the type III secretion systems, specific toxins, and actin polymerization (Francois et al. 2000). This bacterium causes mitochondrial damage with subsequent necrosis in human monocytes-derived macrophages (Koterski et al. 2005). Monocytes from patients carrying disease-associated mutations in the NLR member, cryopirin, exhibit excessive necrotic cell death. *Shigella flexneri* infection also causes cryopyrin-dependent macrophage necrosis with features similar to the death caused by mutant cryopirin. This necrotic death is independent of caspase-1 and IL-1β, and thus independent of the inflammasome (Willingham et al. 2007).

The picture described above indicates a complexity of death receptor-induced necrotic signalling networks that far exceeds that of the simple linear pathways originally indicated by the discovery of the receptor-triggered caspase cascade. This picture also supports the notion that, besides uncontrollable necrosis that may occur following mechanical damage or harsh chemical treatment of tissues, programmed

necrosis may occur with certain molecular events (reviewed in detail Festjens et al. 2006b; Vanlangenakker et al. 2008).

The final fate of necrotic cells as well as apoptotic cells is engulfment by professional or non-professional phagocytes. In the following chapters several authors discuss the molecular mechanisms of necrotic cells engulfment: immunological factors released by necrotic cells (Peter et al., Chap. 3, this Vol.), surface molecules involved in engulfment (Napirei and Mannherz, Chap. 4, this Vol.), and the immunological consequences upon clearance of necrotic cells (Ucker, Chap. 6; Lacy-Hulbert, Chap. 7, this Vol.).

## 1.5 Conclusions

There is mounting interest in apoptotic, necrotic and autophagic cell death types, but a precise description of the morphological and biochemical events in these processes has not yet been derived, and the importance of this cell death type in embryology, ontogeny, physiology and pathophysiology is not yet known. As discussed above, accumulating evidence supports the concept that necrotic cell death is programmed. However, there is no clear biochemical definition of necrotic cell death and consequently no positive biochemical markers that unambiguously discriminate necrosis from apoptosis (Krysko et al. 2008). Unraveling the molecular players and defining the biochemical pathways in necrosis will provide us with powerful and specific methods for identifying necrotic cell and help us to distinguish it positively from other forms of cell death. Future studies aimed at understanding the molecular mechanisms of necrotic cell death will also create many opportunities for development of new therapeutic strategies to modulate necrotic cell death. In this regard, it has been shown that necrostatins, an inhibitor of necrotic cell death, prevent tissue damage in mouse models of cerebral ischemia and myocardial infarction (Degterev et al. 2005; Lim et al. 2007; Smith et al. 2007). We have also discussed the paradoxical roles of autophagy in cell survival and cell death and emphasized that it is not yet clear whether autophagy represents a separate type of cell death or whether it eventually results in apoptotic and necrotic cell death. Future investigations of the molecular mechanisms of all cell death types will yield better insight into the evolution of cell-death programs, their inter-relationships, and their potential for molecular targeting and manipulation in many diseases.

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