Research Paper

The Role of Apoptosis in Cancer Cell Survival and Therapeutic Outcome

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Received 06/03/06; Accepted 09/30/06

Previously published online as a Cancer Biology & Therapy E-publication:
http://www.landesbioscience.com/journals/cbt/abstract.php?id=3456

KEY WORDS
apoptosis, non-apoptotic cell death, cancer cell, tumor response, DNA damage, cancer therapy

ABSTRACT

A number of discoveries have clarified the molecular mechanism of apoptosis, thus clarifying the link between apoptosis and therapeutic outcome. Even though apoptosis is thought to play a major role in anticancer therapy, the clinical relevance of induction of apoptosis remains uncertain, particularly in solid tumors. Induction of apoptosis by anticancer agents has been shown to correlate with tumor response, however, non-apoptotic forms of cell death, such as autophagy and extrinsic senescence, have also been shown to contribute to the overall tumor response. Cellular damage induces growth arrest and tumor suppression by inducing apoptosis, necrosis, and senescence; the mechanism of cell death depends on the magnitude of DNA damage following exposure to various concentrations of anticancer agents. Apoptosis-resistant cells and transduction pathways which inhibit apoptosis can induce non-apoptotic mechanisms of cell death and senescence, thereby preserving the antitumor effect of some anticancer agents. Heterogeneous antitumor responses include various cell types of cell death, depending on the degree of cellular or DNA damage incurred by cancer cells. As a new therapeutic strategy, alternative types of cell death might be exploited to control and eradicate cancer cells. This review discusses the clinical significance of apoptosis, as well as the potential contribution of other types of cell death to overall tumor sensitivity in the hopes that new therapeutic strategies might follow.

INTRODUCTION

Morphological and biochemical characteristics differentiating apoptosis from necrosis were first described in 1972.1 The discovery that Bcl-2 oncoprotein inhibits cell death in non-Hodgkin’s B cell lymphoma, has generated a lot of interest in the molecular mechanism of apoptosis.2,3 Although the importance of physiological cell death in the forms of apoptosis in development was not fully realized, according to the various genetic models apoptosis is critical for deleting structures for controlling cell numbers, and for molding tissue structures.4 Transgenic mice overexpressing Bcl-2 exhibit enlarged brain and an increase in the cell number in the facial nucleus.5 Bcl-2-deficient mice complete embryonic development, but display growth retardation and early mortality postnatally. Thymus and spleen undergo massive apoptotic involution.6 In addition, mature lymphocytes in the mutant mice are incapable of maintaining homeostasis and become vulnerable of apoptotic stimuli such as glucocorticoids and γ-irradiation.7 In contrast, naturally occurring neuronal apoptosis induced by the trophic factor deprivation are reduced in mice deficient in the proapoptotic gene, Bax.8 Further, the Bax deficiency is able to prevent postmitotic neurons from undergoing apoptosis in animals carrying an additional Bcl-xl null mutation, suggesting an intracellular interaction between proapoptotic and antiapoptotic effects in the Bcl-2 protein family.9 Although mice lacking another proapoptotic Bcl-2 member, Bak, develop normally and do not show any abnormality, mice deficient both in Bak and Bax exhibit interdigital webs and imperforate vaginal neurons in the central nervous system.10 The majority of the double knockout mice die perinatally due to the development defects. These findings suggest that apoptosis plays a crucial role in undergoing normal development.

Apoptosis can be induced by a number of factors, including DNA damage and growth factor deprivation, and is characterized by cell shrinkage, blebbing of the plasma membrane, chromatin condensation and DNA fragmentation.1,11 The central component of this process is a proteolytic system involving a family of proteases called caspases. Caspases, also known as cysteine aspartate-specific proteases, are a family of intracellular proteins involved in the initiation and execution of apoptosis. Initiator caspases are able to
activate effector caspases or amplify the caspase cascades by increased activation of initiator caspases. Then the effector caspases cleave intracellular substrates, culminating in cell death. Bcl-2 family members of proapoptotic and antiapoptotic proteins regulate apoptotic cell death by influencing mitochondrial function. Activation of Bax/Bak, which translocate from the cytosol to mitochondria in the formation of oligomers with conformational changes, which are then inserted into mitochondrial membranes, interacting with a voltage-dependent anion channel (VDAC)/adenine nucleotide translocator (ANT) complex. That induces mitochondrial membrane permeabilization (MMP), leading to the release of small molecules, such as cytochrome c, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP) binding protein with low pl (Smac/DIABLO), Omi/HtrA2, apoptosis-inducing factor (AIF), and Endonuclease G (EndoG), through caspase-dependent and -independent pathways. Activation of Bax/Bak and MMP can be inhibited by antiapoptotic Bcl-2 and Bcl-xL. Induction of apoptotic cell death correlates with cellular damage, and there is some communication between the different types of cell death depending on the extent of acquired cellular damage, and there is some communication between the pathways mediating apoptotic and non-apoptotic forms of cell death. Although induction of apoptosis, which results in early cell death, is a key factor influencing chemotherapeutic outcome, non-apoptotic mechanisms of cell death, such as autophagy and necrosis, as well as extrinsic senescence, also influence the overall tumor response to anticancer therapy. The present review discusses the clinical relevance of apoptosis and the potential role of non-apoptotic forms of cell death with regard to therapeutic outcome. In addition, the relationship between apoptotic and non-apoptotic forms of cell death, as well as extrinsic senescence, is discussed with respect to possible strategies for molecular targeting of chemotherapy to enhance the chemotherapeutic effect and overcome drug resistance.

**Programmed Cell Death**

Programmed cell death occurs in both normal development and disease. The earliest studies indicated that cells undergo at least two distinct types of cell death, necrosis and apoptosis. Necrosis is rapid degeneration characterized by cytoplasm swelling, destruction of organelles, and disruption of the plasma membrane, leading to the release of intracellular components and inflammation. A distinct type of cell death, apoptosis, is characterized by cell shrinkage, plasma membrane blebbing, maintenance of organelle integrity, condensation and DNA fragmentation, followed by removal of dying cells by macrophage-mediated phagocytosis. Necrosis is thought to be accidental, uncontrolled degeneration, while apoptosis is thought to represent a cell death program. Given that several pathways of programmed cell death are known to exist, and since cross-talk between the different cell death pathways is known to occur, programmed cell death does not appear to be an isolated process. Three types of programmed cell death have been identified. Type I cell death involves apoptosis. Type II cell death involves autophagy, characterized by an accumulation of autophagic vesicles (autophagosomes) engulfing cytoplasm and/or cytoplasmic organelles, including the mitochondria and endoplasmic reticulum. Autophagy is often observed when there is a demand for massive cell elimination. Autophagic vesicles and their contents are destroyed by the lysosomal system of the same cell. Autophagy either directly executes cell death or occurs secondary to apoptosis. Caspase inhibitors induce autophagic cell death, which occurs as a result of RNA interference with two autophagy genes, ATG7 and Beclin 1. Type III cell death involves non-lysosomal degradation, such as occurs with necrosis, and can be further subdivided into type IIIa (non-lysosomal degradation) or type IIIb (cytoplasmic degradation). The features of these three types of cell death are summarized in Table 1.

**Molecular Mechanism of Apoptotic Cell Death**

Intrinsic apoptotic pathway (mitochondria-initiated pathway). Apoptotic cell death proceeds by cell shrinkage and chromatin condensation, as well as nucleosomal DNA fragmentation. Activation of the caspase family of cysteine proteases gives rise to the characteristic morphological features of apoptosis. Caspase activation occurs following activation of caspase cascades by mitochondrial dysfunction. Forms of mitochondrial dysfunction include MMP, and loss of mitochondrial membrane potential (Δψm, DeltaPsim), which is induced by DNA damage and growth factor deprivation since mitochondria are required for energy production in the form of ATP. MMP has a crucial role in promoting apoptosis, which is mediated by activation of the proapoptotic proteins Bax and Bak, resulting in translocation of Bax/Bak from the mitochondria to the cytoplasm, thereby promoting Bax/Bak oligomerization. Oligomers of Bax or Bak are inserted into the mitochondrial outer membrane to form the mitochondrial transition pore (MTP) in combination with a VDAC/ANT, leading to the release of a number of proapoptotic small molecules, such as cytochrome c. Smac/DIABLO, Omi/HtrA2, AIF, and EndoG, from the intermembrane space into the cytoplasm. Cytochrome c binds to Apaf-1, recruiting procaspase-9 and forming an apoptosome in the presence of ATP/dATP for activation of the initiator caspase, caspase-9. Caspase-9 activates caspase-3, initiating a cascade in which caspase-3
c-Jun NH2-terminal kinase (JNK) is required for the release of mitochondrial cytochrome c and apoptosis, and that the Bax subfamily of Bcl-2-related proteins is essential for JNK-dependent apoptosis. Activated JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3, a cytoplasmic anchor of Bax. Phosphorylation of 14-3-3 led to dissociation of Bax from this protein. Although JNK can be activated in response to treatment with anticancer drug, the JNK pathway is activated and a prolonged JNK activation was maintained in drug-sensitive cells, whereas only a transient activation was detected in drug-resistant cells. In fact, inhibition of JNK activity by transfection with a dominant negative allele of JNK blocked anticancer drug-induced apoptosis significantly in drug-sensitive cells. Further, the persistent activation of JNK resulted in hyperphosphorylation of the c-Jun transcription factor, which in turn stimulated the transcription of an immediate downstream target, the death inducer Fas ligand (FasL). Although drug-resistant cells were associated with failure to upregulate FasL, selective stimulation of the JNK pathway by adenovirus-mediated delivery of recombinant M KK7 led to sensitization to apoptosis through reactivating FasL expression. Thus, JNK/FasL-signaling pathway might play an important role in mediating anticancer drug-induced apoptosis, and the duration of JNK activation might be critical in determining whether cells survive or undergo apoptosis. In addition, despite activation of the antiapoptotic IkB kinase (IKK)/NFκB pathway, TNF-α is able to induce apoptosis in sensitive cells, such as human breast carcinoma MCF-7 and fibroblast cells. TNF-α-induced apoptosis is suppressed by inhibition of the JNK pathway but promoted by its activation. Furthermore, activation of JNK by TNFα was transient in TNFα-insensitive cells but prolonged in sensitive cells. Conversion of JNK activation from prolonged to transient suppressed TNF-α-induced apoptosis.

Caspase-12 is a cystein caspase belonging to a caspase-1 subfamily, which is located in the endoplasmic reticulum (ER). The ER was shown to be another center of regulation for cell death. In this pathway, caspase-12 is thought to be an initiator caspase, which plays a very important role. Although the apoptotic stimuli such as serum deprivation and TNF treatment do not cause activation of caspase-12, agents inducing ER stress such as brefeldin A (an inhibitor of ER/Golgi transport) and tunicamycin (an inhibitor of N-linked glycosylation in the ER) initiate caspase-12 cleavage and subsequent activation. Calpain, another cystein protease, is responsible for cleaving procaspase-12 to generate its active caspase-12. Since caspase-12 also has a caspase recruitment domain, through which caspase-9 and Apaf-1 interact and form an apoptosome complex, it may be possible that caspase-12 is activated by its association with an Apaf-1-like protein. In the downstream apoptotic pathway of ER-stress-mediated cell death, JNK is translocated to the mitochondrial membrane, which stimulates the phosphorylation of Bim, a BH3-only Bcl-2 protein, resulting in Bax-dependent cytochrome c release.

Extrinsic apoptotic pathway (death receptor-dependent pathway). MMP as a result of Bax/Bak activation promotes apoptotic cell death, and the BH-3 only protein, Bid, activates Bax/Bak following activation by caspase-8. Caspase-8 activation occurs through activation of the death receptors FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Activation of Fas, DR4, and DR5 by binding of FasL and TRAIL recruits TNFR1-associated protein (TRADD) and FADD, which leads to the formation of an activatory death-inducing signaling complex (ASC) that bridges between the death receptor and the caspase-8.

Table 1 Differences in the features of apoptotic, necrosis and autophagy

<table>
<thead>
<tr>
<th>Feature/Type of Cell Death</th>
<th>Apoptosis</th>
<th>Necrosis</th>
<th>Autophagy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphological change</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Chromatin condensation</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Nuclear fragmentation</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cell shrinkage</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytoplasmic vacuoles</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mitochondrial swelling</td>
<td>±</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Genomic change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internucleosomal</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TUNEL staining</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caspase activity Caspase-3 activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP cleavage</td>
<td>(85 kDa)</td>
<td>(50-62 kDa)</td>
<td>-</td>
</tr>
<tr>
<td>ATP requirement</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Inflammation</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Exteriorization of phosphatidylserine</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Inhibitory action</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zVAD-fmk</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>+</td>
<td>± (Partial)</td>
<td>-</td>
</tr>
<tr>
<td>Actinomycin D/cycloheximide</td>
<td>(Sometimes)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
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death domain protein (TRADD), as well as Fas-associated death domain protein (FADD) and procaspase-8, which together form a death-inducing signaling complex (DISC), resulting in activation of caspase-8. Following TRAIL activation, FADD is recruited after TRADD dissociates to form a complex with receptor-interacting protein (RIP) and TRAF2, which mediates cell death and survival through NFκB and JNK1, respectively. Caspase-8 causes proteolytic cleavage of Bid, resulting in the formation of tBid, which activates the Bax/Bak-mediated mitochondrial pathway. Bid is also activated by granzyme B, lysosome, and calpain. JNK activates Bid (jBid), which releases Smac/DIABLO following TNF-induced caspase-8 activation, leading to apoptosis. Marked activation of death receptor-dependent apoptotic signals occurs in the context of mild cellular injury (type II cells). Marked cellular damage results in activation of caspase-3, since caspase-8 can directly activate caspase-3 in the absence of mitochondrial pathway amplification (type I cells). Thus, in type I cells caspase-3 becomes directly activated by caspase-8, whereas in type II cells caspase-3 activation involves activation of caspase-9 through the mitochondrial pathway. Even though activation of the receptor-dependent apoptotic pathway, with or without amplification, depends on the extent of cellular damage, the magnitude of cellular damage can determine whether there is direct activation of caspase-3 by caspase-8, or merely amplification of the mitochondrial pathway, even in type II cells (Fig. 2).

Given that a balance between cell proliferation and cell death is required for maintaining tissue homeostasis, excess or lack of apoptosis leads to disease pathogenesis such as autoimmune disease and cancer. The FasL and TRAIL-mediated cytotoxicity in immune effector cells plays an important role in immune surveillance. In the Fas/FasL system, the study of two spontaneous recessive mutations in the Fas and FasL genes in mice, lpr (lymphoproliferation) and gld (generalized lymphoproliferative disease) showed systemic autoimmunity, lymphadenopathy, and splenomegaly with a marked accumulation of CD4-CD8- T cells. Impairment of the Fas/FasL system causes increased resistance of T cells to activation-induced apoptosis, resulting in mature CD4-CD8- T cells can not be eliminated by the system, which accumulates in lymph nodes and spleen. These findings suggest important roles of the Fas/FasL system in development of T cells as well as cytotoxic T lymphocyte-mediated cytotoxicity. In the TRAIL-R/TRAIL system, a recent study showed the evidence supporting a physiological role of TRAIL as a tumor suppressor. TRAIL is constitutively expressed in a large number of liver NK cells, which exerts as the antimetastatic function of liver NK cells against TRAIL-sensitive tumor cells.

Antia apoptotic pathway. Even though Bax/Bak-mediated MMP and the subsequent release of small molecules are essential for caspase-dependent and caspase-independent apoptotic cell death, this can be inhibited by the antia apoptotic proteins, Bcl-2 and Bcl-xL. Antia apoptotic Bcl-2 family members are major inhibitors of apoptotic

Figure 1. Apoptotic and non-apoptotic cell death pathways mediated by mitochondrial membrane permeabilization in response to DNA damage. Proapoptotic small molecules, including cytochrome c, Smac/DIABLO, Omi/HtrA2, apoptosis-inducing factor (AIF), and endonuclease G (EndoG), are released via the mitochondrial permeability transition pore, which is regulated by an anion channel, voltage-dependent anion channel (VDAC), and adenine nucleotide translocase (ANT), resulting in cell death. The caspase-dependent pathway is activated by the release of cytochrome c and Smac/DIABLO and the caspase-independent pathway is executed by AIF, autophagy, and other mechanisms of cell death. The overall tumor response to chemotherapy is the result of the accumulative effect of these various cell death pathways. PARP-1, poly (ADP-ribose) polymerase-1; ICAD, inhibitor of caspase-activated DNase; CAD, caspase-activated DNase.
resulting in DISC formation, leading to activation of caspase-8. Response to minor damage usually involves amplification of the mitochondrial pathway, in which caspase-8 cleaves Bid (tBid), thereby promoting oligomerization of Bax or Bak, as part of the intrinsic pathway (type II). Major damage usually results in direct activation of caspase-8, which in turn activates caspase-3, thereby producing a cell type-dependent response in severely damaged cells. Non-transcriptional activation of p53 induces binding to Bcl-2 and Bcl-xL, after which released BH3-only protein facilitates production of a cell type-dependent response in severely damaged cells.

Figure 2. Schematic representation of the extrinsic and intrinsic apoptotic cell death pathways. Activation of Fas and DR4/5 by binding of Fas L and TRAIL recruits FADD and procaspase-8, resulting in DISC formation, leading to activation of caspase-8. Response to minor damage usually involves amplification of the mitochondrial pathway, in which caspase-8 cleaves Bid (tBid), thereby promoting oligomerization of Bax or Bak, as part of the intrinsic pathway (type II). Major damage usually results in direct activation of caspase-8, which in turn activates caspase-3, thereby producing a cell type-dependent response in severely damaged cells. Non-transcriptional activation of p53 induces binding to Bcl-2 and Bcl-xL, after which released BH3-only protein facilitates production of a cell type-dependent response in severely damaged cells. Non-transcriptional activation of p53 induces binding to Bcl-2 and Bcl-xL, after which released BH3-only protein facilitates production of a cell type-dependent response in severely damaged cells.
Given that NFκB transcription factors block apoptosis induced by TNFα, the antiapoptotic activity of NF-κB is also crucial for tumorigenesis and cancer chemoresistance. This inhibitory activity of NFκB in TNFα-induced apoptosis depends upon transcriptional upregulation of blockers of the JNK cascade such as the caspase inhibitor XIAP, the zinc-finger protein A20, and the inhibitor of the MKK7/JNK2 kinase Gadd45B/Myd118. Since MKK7/JNK2 is a specific and essential activator of JNK as a target of Gadd45B, Gadd45B binds to MKK7 directly and blocks its catalytic activity, thereby providing a molecular link between the NFκB and JNK pathways.108 In addition, NF-κB decreases accumulation of ROS induced by TNFα, and this antioxidant effect of NF-κB is also critical for inhibition of TNFα-induced JNK activation. Suppression of ROS by NFκB is mediated by feritin heavy chain (FHC) that is the primary iron-storage mechanism in cells, and possibly, by the mitochondrial enzyme Mn2+ superoxide dismutase (Mn-SOD).109 FHC is as an essential mediator of the antioxidant and protective activities of NFκB, and FHC-mediated inhibition of JNK signaling depends on suppressing ROS accumulation and is achieved through iron sequestration. Thus, induction of FHC and Mn-SOD indicates an additional effect by which NFκB inhibits proapoptotic JNK signaling. The antiapoptotic activity of NFκB involves suppressing the accumulation of ROS and controlling the activation of the JNK cascade.

Heat shock proteins (HSPs) also have important roles in cell growth and apoptosis.110 A number of studies have shown HSP superfamily proteins, including hsp90, hsp70, hsp60 and hsp27, to regulate the biological behavior of cells, particularly Fas pathway signal transduction leading to apoptosis, as well as the JNK and caspase pathways, partly by functioning as molecular chaperones. In fact, overexpression of HSP27 and HSP70 inhibits the function of AIF proteins and apoptosomes, and inhibits caspase activation leading to apoptosis.110-113 Overexpression of apoptosis-inhibitory HSPs, particularly HSP27 and HSP70, can increase the tumorigenicity of tumor cells.

**NON-APOPTOTIC CELL DEATH**

**Necrosis.** Necrosis is uncontrolled cell death induced by a loss of ATP or impairment of the mitochondrial membrane pump. Necrotic cell death most commonly involves osmotic swelling of the cell membrane and organelles, with extraction of contents, as well as an inflammatory response.114 Necrosis can be induced by apoptosis (secondary necrosis). Although death receptors primarily induce apoptosis, there is growing evidence to suggest that these receptors also possess a variety of non-apoptotic functions. TNF and TRAIL induce apoptotic and non-apoptotic cell death.115 TNF effectively induces cell death bearing morphological resemblance to necrosis in wild-type MEFs under caspase-inhibited conditions. Tumor necrosis factor receptor (TNFR) I mediates TNF-induced necrosis, and RIP, FADD, and TRAF2 are critical components of TNF-induced necrosis.116 Inhibitors of NFκB facilitate TNF-induced necrosis, suggesting that NFκB suppresses necrosis.117 During TNF-induced necrosis, significantly elevated cellular levels of ROS were noted in wild type, but not RIP−/−, TRAF2−/− or FADD−/− cells, suggesting that RIP, TRAF2, and FADD are crucial mediators of ROS production in TNF-induced necrosis.116 Several anticancer drugs also preferentially induce necrosis, rather than apoptosis. Apoptosis is induced by the topoisomerase II inhibitor, etoposide, while necrosis is induced by ethacrynic acid and cytochalasin B in human HeLa cervical carcinoma cells.118 Treatment with DNA alkylating agents, such as cisplatin, as well as other cytotoxic agents, produces the same degree of necrosis in cells regardless of whether they have apoptotic defects, in the absence of p53 or Bax and Bak.119 Although ATP depletion causes necrotic cell death in hypoxic and anoxic regions of solid tumors, the importance of necrosis in enhancing the tumor response is less clear than that of apoptosis.

Hypoxic conditions exist within solid tumors, such as pancreatic and gastric carcinoma, yet cancer cells survive and replicate within this environment. Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), a proapoptotic member of the Bcl-2 family, is a regulator of hypoxia-induced cell death, further regulated by hypoxia inducible factor 1 (HIF-1). BNIP3 induces a loss of DeltaPsim, resulting in necrotic cell death without the release of cytochrome c.120,121 However, frequent aberrant methylation of the 5′ CpG island and silencing or downregulation of the BNIP3 gene are detected in 66% of primary colorectal and 49% of primary gastric tumors, but not in normal tissue samples collected from adjacent areas, suggesting that inactivation of BNIP3 likely plays a key role in the progression of some gastrointestinal cancers.122 Furthermore, aberrant DNA methylation of the 5′ CpG island and histone deacetylation play key roles in silencing BNIP3 expression in cell lines of haematopoietic tumors, such as acute lymphocytic leukemia, multiple myeloma, and Burkitt’s lymphoma.123 In addition, hypoxia can induce antiapoptotic proteins, such as IAP-2, and downregulate the proapoptotic protein Bax. Thus, even though induction of apoptosis is a response to hypoxia, cancer cells can escape hypoxia-induced cell death, and selection for resistance to hypoxia-induced cell death probably creates more aggressive phenotypes with decreased responsiveness to treatment.

**Autophagy.** Autophagy is a degradative mechanism, which primarily mediates the recycling and turnover of various cytoplasmic eukaryotic cell constituents. Autophagy is activated in response to nutrient starvation, differentiation, and developmental triggers. It is an adaptive process responding to metabolic stress that results in degradation of intracellular proteins and organelles.124 The process of autophagy involves formation of a double-membrane vesicle (autophagosome) in the cytosol that engulfs organelles and cytoplasm and then fuses with the lysosome to form the autolysosome where the contents are degraded and recycled for protein and ATP synthesis.125 The formation of the autophagosome is mediated by a series of autophagy-specific genes (Atg).126 Under normal physiological conditions, autophagy occurs at basal levels in most cells, contributing to the routine turnover of cytoplasmic components. It can promote cell adaptation and survival during stress such as starvation, but under some conditions cells undergo death by excessive autophagy. Genetic screening of yeast has considerably improved our knowledge regarding the molecular signaling behind autophagy, and a number of autophagic genes have been identified. Some of the mammalian orthologs to these genes have been identified. Studies involving Beclin 1, the mammalian ortholog of yeast Atg6, gave the first indication linking dysfunctional autophagy with tumorigenesis.127 Beclin 1 is required for autophagosome formation and has been suggested to be a haploinsufficient tumor suppressor gene. Beclin 1−/− mice showed a high incidence of spontaneous tumors such as lung cancer, hepatocellular carcinoma, and lymphoma,129,130 and Beclin 1 is monoaicetically deleted in 40–75% of sporadic cases of human breast, ovarian, and prostate cancer, while disruption of both alleles causes increased cellular proliferation and inhibition of autophagy in vivo.131 In addition, the observation that other tumor...
suppressors, such as death-associated protein kinase (DAP-kinase)\(^{132}\) and PTEN, induce autophagic cell death pathways implicates defective autophagy in tumorigenesis. Thus, defects in lysosome-mediated autophagic protein degradation enhance carcinogenesis. Nevertheless, autophagic cell death is induced by γ-irradiation and chemotherapy, both of which are linked to activation of the lysosomal pathway.

Autophagy can promote cell adaptation and survival, but under some conditions it leads to cell death. In fact, a number of studies have reported that autophagy, or autophagic cell death, is activated in cancer tissues derived from such as breast, brain, colon, prostate and ovary in response to various anticancer therapies.\(^{135}\) Tamoxifen, an antiestrogen agent induces autophagic cell death in breast cancer cells,\(^{134}\) and this has been shown to be associated with downregulation of Akt.\(^{135}\) Similarly, the antitumor effects of anti-PDGF antibody are mediated by phosphorylated Akt, resulting in inhibition of PDGF and the development of acidic vesicular organelles, as well association of microtubule-associated protein light chain 3 (LC3) with the autophagosome membrane, both of which characterize autophagy in malignant glioma cells.\(^{136}\) Temozolomide, a DNA alkylating agent induces autophagy in malignant glioma cells.\(^{137}\) Other anticancer agents including γ-irradiation induce autophagy or autophagic cell death in cancer cells such as breast, colon, and prostate cancer.\(^{138}\) The histone deacetylase (HDAC) inhibitors, butyrate and suberoylanilide hydroxamic acid (SAHA), both induce mitochondrial-mediated apoptosis and caspase-independent autophagic cell death in multiple human cancer cell lines.\(^{139}\) Ceramide controls autophagy, a major lysosomal catabolic pathway. For example, exogenous C2-ceramide stimulates proteolysis and the accumulation of autophagic vacuoles, key features of autophagy, in human colon cancer HT-29 cells.\(^{135}\) In addition, C2-ceramide stimulates expression of the autophagy gene product Beclin 1. Ceramide also mediates the tamoxifen-dependent accumulation of autophagic vacuoles in human breast cancer MCF-7 cells.\(^{135}\) The natural product arsenic trioxide (\(\text{As}_2\text{O}_3\)) induces autophagy in malignant glioma cells,\(^{140,141}\) and the natural product resveratrol induces in ovarian cancer cells.\(^{142}\) Although \(\text{As}_2\text{O}_3\) induces autophagy, this is likely due to activation of Bnip3 since \(\text{As}_2\text{O}_3\)-induced autophagic cell death is enhanced by exogenous expression and transfection of Bnip3.\(^{140}\) Caspase inhibitors do not halt \(\text{As}_2\text{O}_3\)-induced cell death.\(^{141}\) Rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), induces autophagy in suppressing the proliferation of malignant glioma cells.\(^{143}\) Interestingly, since CD4+ T cells classically recognize antigens that are subsequently endocytosed and processed in lysosomes for presentation on major histocompatibility complex (MHC) class II molecules, lysosomal processing after autophagy might enable MHC class II-restricted surveillance of long-lived endogenous antigens, including nuclear proteins.\(^{144,145}\)

Autophagy is controlled by the mTOR pathways. The mTOR pathway integrates the cellular response to growth factors and nutrients through regulation of protein synthesis. In mammals, mTOR is regulated by growth factors through the class I PI3K/Akt signaling pathway,\(^{146}\) and by the downregulation of nutrient transporters during growth factor withdrawal.\(^{147}\) Given that existence of cross-talk between PI3K/Akt pathway and the Ras/extracellular signaling pathways was suggested, epidermal growth factor activation through extracellular signal-regulated kinases and Akt blocked the induction of autophagy, whereas PTEN, a negative regulator, stimulated autophagy in HT-29 colon cancer cells.\(^{148}\) Akt is located downstream of class I PI3K, that activates the kinase mTOR,\(^{149}\) leading to suppression of autophagy. Class I and class III PI3K show differential regulation of autophagy. The class I PI3K signal, which is activated through the growth factor receptor, inhibits autophagy, whereas class III PI3K promotes the sequestration of cytoplasmic components.\(^{150}\) PTEN dephosphorylates and thereby inhibits class I PI3K, which suppress Akt activity and allows autophagy to be induced.\(^{148}\) PTEN is often deleted and mutated in various cancers, resulting in constitutive activation of the Akt pathway, and inhibit autophagy.\(^{148}\)

Despite the fact whether cancer cells are eliminated by autophagy remains controversial, the presence of autophagic vacuoles in dying cancer cells after anticancer treatment indicates that they undergo autophagy-related cell death, but they are not always an association between autophagy and cell death. When anticancer treatments induce damage of organelles in cancer cells, autophagy might initially triggered to protect cells by sequestering and degrading the damaged organelles. However, once a critical threshold of intracellular is reached, autophagy might serve to remove the damaged cells from a tissue by inducing cell death.\(^{151}\) Given that autophagic cell death is characterized by degradation of the Golgi apparatus and endoplasmic reticulum before nuclear destruction, the occurrence of caspase-independent cell death with an increased autophagic vacuoles might be a hallmark of autophagic cell death. These idea may be supported by the findings that although IL-3-dependent Bax\(^{-/-}\) Bak\(^{-/-}\) cells eventually die by autophagic cell death,\(^{147}\) because of excessive self-consumption and bioenergetic failure, at any point before death, the addition of growth factor reverses the catabolic process and maintains cell viability. These observations are consistent with the concept that autophagy is a self-limited survival strategy, rather than a primary or irreversible death execution program. Nevertheless, given the uncertain physiological relevance of autophagic gene-dependent cell death in zVAD-treated cells or in Bax\(^{-/-}\) Bak\(^{-/-}\) cells, it seems that autophagy is a physiologically important cause of cell death.

**Senescence.** Since normal somatic cells have a limited life span and cannot proliferate indefinitely in culture, after a certain number of doublings, called the Hayflick limit, there is an irreversible cessation of proliferation. This is called replicative senescence (intrinsic senescence), and is due to shortening of telomeres after each cell division.\(^{152}\) Telomerase, which prevents the loss of telomeres, is thought to inhibit cellular senescence since it is overexpressed in cancer cells and absent or present at low levels in normal cells.\(^{153}\) Given that escape from senescence in cancer cells is linked to inactivation of the tumor suppressor p53, as well as constitutive activation of telomerase, cancer cells are less sensitive to senescence induced by physiological conditions. However, exogenous stress, such as UV light, irradiation, hypoxia, and anticancer drugs, induces senescence in cancer cells (extrinsic senescence) independent of telomerase expression and telomere shortening. This is likely due to enhanced expression of the cell cycle inhibitors, p16\(^{\text{INK}}\), p21\(^{\text{WAF}}\), and p27\(^{\text{KIP}}\), as well as disruption of lysosomal function through enhanced activity of senescence-associated beta galactosidase (SA-β-Gal).\(^{154}\)

Reactive oxygen species are associated with senescence.\(^{155}\) Cells undergoing senescence express higher levels of ROS than normal cells. Furthermore, oxidative stress and hypoxia can arrest human fibroblasts in a state that resembles senescence, suggesting a relationship between oxidative damage and senescence.\(^{156}\) Overexpression of p53 has been observed in association with an accumulation of ROS,
presumably due to the effects of prooxidant agents on the transcription of p53. Activation of the CDK inhibitor p21WAF1 by p53 results in increased levels of ROS in normal and tumor cells, the levels of which are proportional to the level of p21WAF1. Accumulation of ROS is not a general consequence of cell cycle arrest, since another CDK inhibitor, p16INK4A, does not increase ROS levels. Rather, an accumulation of ROS leads to permanent cell cycle arrest following induction by p21WAF1. In contrast to that of p21WAF1, p16INK4A-induced growth arrest is reversible, perhaps due to a lack of ROS accumulation.

In short, these findings suggest that ROS accumulation is responsible for permanent growth arrest/senescence induced by p21WAF1, which is known to play a crucial role in tumor suppression.

Cancer cells are susceptible to irreversible growth arrest following exposure to irradiation and chemotherapeutic agents, suggesting that they respond similarly to normal cells to exogenous stimuli by undergoing senescence. The precise mechanism by which anticancer drugs induce cellular senescence in response to DNA damage beyond a certain threshold is controversial. DNA damaging agents, such as topoisomerase inhibitors and alkylating agents, induce double strand DNA breaks producing lethal DNA damage. Normally, cell cycle arrest in response to DNA damage occurs at check-points enabling DNA repair prior to progression of the cell cycle, however, if the damage is too extensive, cells commit to senescence or apoptosis. Thus, growth inhibition varies depending on the drug concentrations used in vitro. Low concentrations of anticancer agents reduce the incidence of apoptosis and increase the rate of extrinsic senescence, which is associated with G1-S checkpoint arrest and stabilization of p53 leading to increased transcription of p21WAF1 and growth arrest. Thus, irreversible growth arrest and senescence can be achieved with lower drug concentrations than those required to induce apoptosis. Greater drug concentrations result in apoptosis. Of importance, the significance of extrinsic senescence with regard to therapeutic outcome following chemotherapy has been demonstrated using a primary murine lymphoma model. Tumors in which drug-induced senescence is observed have a much better prognosis than tumors in which senescence is not observed due to p53 and p16INK4A mutations, which promote tumorigenesis and drug resistance, in part, by disabling apoptosis. Thus, extrinsic senescence induced by various death triggers in cancer cells with sublethal damage might inhibit tumor proliferation by enabling cells to escape senescence.

When only a few telomeres are short, cells form end-associations, leading to a DNA damage signal resulting in intrinsic senescence (M1 stage). In the absence of cell-cycle checkpoint pathways (p53 and/or p16/Rb), cells bypass M1 senescence and telomeres continue to shorten eventually resulting in crisis (M2 stage). M2 is characterized by many ‘uncapped’ chromosome ends, chromosome breakage mimicking mitotic catastrophe and a high fraction of apoptotic cells. Mitotic catastrophe, a form of cell death that results from abnormal mitosis and leads to the formation of interphase cells with multiple micronuclei, which is induced by different classes of cytotoxic agents, but the pathways of abnormal mitosis differ depending on the nature of the inducer and the status of cell-cycle checkpoints. Mitotic catastrophe results from a combination of deficient cell-cycle checkpoints and cellular damage-two subtypes of mitotic catastrophe can be distinguished. First, mitotic catastrophe can kill the cell during the metaphase, in a p53-independent fashion. Second, mitotic catastrophe can occur after failed mitosis, during the activation of the polyploidy checkpoint, in a partially p53-dependent fashion. Failure to arrest the cell cycle before or at mitosis triggers an attempt of aberrant chromosome segregation, which culminates in the activation of the apoptotic default pathway and cellular demise. Cell death occurring during the metaphase transition is characterized by the activation of caspase-2 and MMP, which can be activated in response to DNA damage with the release of cell death effectors such as AIF and cytochrome c. Although the morphological aspect of apoptosis may be incomplete, this form of cell death indeed constitutes the biochemical hallmarks of apoptosis.

Suppression of caspase-2 by RNA interference or pseudosubstrate inhibitors as well as blockade of the MMP prevent the mitotic catastrophe and allow cells to further proceed the cell cycle beyond the metaphase, leading to asymmetric cell division. Mitotic catastrophe thus may be conceived as a molecular device that prevents aneuploidy, which may participate in oncogenesis.

When apoptosis was suppressed by overexpression of Bcl-2, many drug-induced cancer cells were died through mitotic catastrophe alone, and the inhibition of radiation-induced apoptosis in HeLa and NIH 3T3 cells also does not protect from radiation-induced cell death because it leads to both mitotic catastrophe and senescence.

Prolonged cell cycle arrest (senescence) may be an important phenomenon for the determination of tumor sensitivity in DNA-damaged cells. Indeed, cell cycle arrest in the G1 and G2 phases commits to either cell death or cell survival after DNA damage; in contrast, in the case of escaped cells from the G1/G2 checkpoint which did not receive lethal damage, these cells might be undergoing senescence or non-apoptotic cell death. Not only apoptosis but also premature senescence as drug-inducible programs may influence treatment outcome. In this point, survival of the senescent cells after DNA damage is another important factor for determining tumor sensitivity because tumor cells are consistent with heterogeneous populations, and G1 populations in hypoxic lesions are major components of solid tumors. Furthermore, previous studies reported that despite resistance to apoptosis in senescent T-lymphocytes and fibroblasts mediated by serum withdrawal, autophagic cell death was induced in vitro in senescent cells by the accumulation of autophagosomes. The increased sensitivity to senescent cells by autophagic cell death may be an important phenomenon for anticancer treatment.

**POTENTIAL LIMITED ROLE OF APOPTOTIC CELL DEATH**

Apoptosis is thought to significantly contribute to tumor response following chemotherapy. A number of in vitro and in vivo studies indicate a correlation between apoptosis and tumor response following chemotherapy. However, the magnitude of apoptotic cell death following lethal dose administration does not always correlate with overall tumor response, as assessed by MTT and clonogenic assay. In fact, defects in apoptosis in several knockout models, including p53-/-, Apaf-1-/-, caspase-3-/-, and Bax-/-Bak-/- cells, result in non-apoptotic forms of cell death, such as necrosis and autophagy. Likewise, inhibition of apoptosis by pancaspase inhibitors does not prevent anticancer drug-induced cell death. Clonogenic assay findings regarding the role of non-apoptotic forms of cell death in overall tumor sensitivity, indicate that non-apoptotic forms of cell death occur as a late response, while apoptosis occurs as an early response, to cellular injury. Thus, both apoptotic and non-apoptotic forms of cell death likely contribute to the overall tumor response to chemotherapy, suggesting that if apoptotic cell death indeed constitutes the biochemical hallmarks of apoptosis, then suppression of apoptosis may improve tumor sensitivity.
death is impaired, cell death might occur via non-apoptotic mechanisms of cell death. Thus, the magnitude of early induction of apoptosis might not determine overall tumor sensitivity in cancer cells with defects in apoptosis. The ability to differentiate between induction of apoptosis or necrosis by an anticancer agent, particularly in the early stages of cell death, might be key to determining its clinical efficacy.\textsuperscript{118}

Although several anticancer drugs and irradiation induce non-apoptotic forms of cell death, such as autophagy and necrosis, as well as apoptosis, the interplay between apoptosis and non-apoptotic forms of cell death remains unclear. Even among cancer cells with similar drug sensitivities, various forms of cell death, such as apoptosis, necrosis, autophagy, and extrinsic senescence, are observed, depending on the drug concentrations used. Heterogeneous tumor cell populations display different drug sensitivities and are also susceptible to more than one type of cell death. Three possible mechanisms of cross-talk between cell death pathways in response to death triggers exist (Fig. 3). The first is that one pathway might inhibit another, as occurs with growth factor deprivation. Induction of autophagy by growth factor deprivation inhibits apoptosis for cell survival in IL-3-dependent Bax\textsuperscript{-/-}Bak\textsuperscript{-/-} cells.\textsuperscript{147} Despite an abundance of extracellular nutrients, growth factor-deprived cells maintain ATP production by catabolism of intracellular substrates through autophagy, suggesting that growth factors are required to direct the utilization of exogenous nutrients to maintain cell viability. A second possibility is that one pathway might be inhibitory when upregulated, as is observed in death receptor-dependent cell death. Although TNF-\alpha- and TRAIL-induced cell death is normally characterized by a combination of apoptosis and necrosis, apoptosis is inhibited by enhancement of necrotic cell death by caspase inhibitors.\textsuperscript{174} Furthermore, activation of RIP, a serine-threonine kinase and JNK induces cell death morphologically resembling autophagy, which is further induced by caspase-8 inhibition.\textsuperscript{33} In contrast, 3-methyladenine (3-MA), which inhibits autophagy, increases the sensitivity of HT-29 cells to apoptosis induced by sulindac sulfide, a non-steroidal antiinflammatory drug which inhibits cyclooxygenase enzymes.\textsuperscript{175} Furthermore, HT-29 cells made to overexpress GTPase-deficient G\textalpha3 protein, have low rates of autophagy and are more sensitive to sulindac sulfide-induced apoptosis than parental HT-29 cells.\textsuperscript{175} Differences in COX-2, Bcl-2, Bcl-x\textsubscript{L}, Bax, and Akt expression are not observed between mutant G\textalpha3 protein-expressing and parental HT-29 cells, however, increased cytochrome c release is observed from mutant G\textalpha3 protein overexpressing cells. It is also possible that apoptosis and non-apoptotic forms of cell death function independently of one another. Treatment of Bax\textsuperscript{-/-}Bak\textsuperscript{-/-} cells with etoposide inhibits apoptosis, but not non-apoptotic forms of cell death, such as autophagy, which is regulated by Bcl-x\textsubscript{L}.\textsuperscript{22} Similarly, treatment of a T-lymphoblastic leukaemic cell line (CCRF-CEM) with TNF\alpha induces apoptosis, preceded by autophagy.\textsuperscript{176} Prevention of autophagy by 3-MA does not inhibit TNF\alpha-induced apoptosis.

Conversion from apoptosis to other types of cell death results in autophagy, as well as senescence, since caspase inhibitors do not protect cancer cells from death in the same way as cytotoxic agents, but rather expose cells to other forms of cell death through prevention of apoptosis. Although doxorubicin results in at least three distinct types of cell death, differential drug sensitivity of cancer cells might result in cell death by extrinsic senescence, even following exposure to a lethal dose of doxorubicin which would normally result in apoptosis.\textsuperscript{24} The pancaspase inhibitor Q-VD-OPH greatly inhibits doxorubicin-induced apoptosis along with a decrease in caspase-3 activation, even though it does not protect cells from drug toxicity. A combination of doxorubicin and Q-VD-OPH results in increased expression of p21\textsuperscript{WAF1} and SA-\textbeta-Gal activity, however, it does not alter Akt activity.\textsuperscript{24} These findings suggest that inhibition of apoptosis might lead to increased expression of cell cycle inhibitors and cellular senescence. Thus, cancer cells might switch from apoptosis to alternative pathways of cell death as a result of defects in apoptosis following DNA damage. However, the significance of this with regard to overall tumor response and ability to overcome drug resistance remains unclear.
While p53 mutations and overexpression of Bcl-2 usually inhibit apoptosis, they do not enhance overall survival or prevent cellular proliferation, thereby raising the question as to whether apoptosis is a critical determinant of chemotherapeutic outcome and survival in cancer patients. A number of studies suggest that p53 mutations or p53 overexpression do not affect prognosis with or without treatment, compared to wild type p53. In addition, inhibition of apoptosis and overexpression of Bcl-2 does not protect cancer cells from cell death induced by chemotherapeutic agents, indicating that apoptosis is replaced by senescence of other forms of cell death. Thus, induction of apoptosis may not be the sole predictor of overall tumor sensitivity to anticancer treatment. Cancer cells resistant to apoptosis may have reduced susceptibility to drug-induced cell death, but still experience growth arrest in response to anticancer drugs, which promote both apoptosis and senescence in cells lacking apoptotic defects. Despite their effects on apoptosis, all anticancer drugs inhibit tumor cell proliferation, which contributes to the overall tumor response and therapeutic outcome of cancer therapy. The potential benefit of irreversible growth arrest through the use of less toxic drug concentrations remains to be determined.

**CONCLUDING REMARKS**

Evidence regarding the molecular mechanism of apoptosis has contributed to our understanding of anticancer drug-induced cell death. It is likely that induction of apoptosis determines the early tumor response to chemotherapy. A number of preclinical studies and some clinical studies show that the magnitude of induction of apoptosis is associated with tumor response, and that resistance to apoptosis leads to drug resistance. Furthermore, targeted therapy against apoptosis-related proteins and subsequent effects on drug resistance are being studied (Table 2). However, the exact contribution of apoptosis to overall tumor sensitivity remains unclear. For one, it is difficult to assess the magnitude of induction of apoptosis using clinical samples. A recent report using nuclear magnetic resonance spectroscopy shows different times to induction of apoptosis and necrosis, and further demonstrates the contribution of non-apoptotic forms of cell death, such as necrosis, to the overall tumor response.

Despite the fact that various types of cell death, including apoptosis, necrosis, autophagy, and senescence are involved in response to anticancer treatment, it seems that detection and its quantification of cell death are not so easy in clinical sample as a routine work. In fact, the fragmented DNA can be labeled by TUNEL method, and senescence is also detected by activity of SA-β-Gal. Further, the difference between apoptosis and necrosis can be distinguished by flow cytometric analysis, which is labeled with Annexin V and propidium iodide (PI) in hematological malignancies. In contrast, methods for monitoring autophagy have been very limited and unsatisfactory. The most standard method is conventional electron microscopy, and some biochemical methods have been utilized to measure autophagic activity. Recently, the molecular basis of autophagosome formation has been extensively studied using yeast cells. LC3 protein, which is an attractive marker of autophagosomes, revealed a relatively low expression level in tissue and cultured cells, but could be detected via immunohistochemistry in liver from GFP-LC3 transgenic mice. LC3 immunostaining can be used as an alternative detection method for autophagy in situ. Thus, although there are still limitations of methods for distinguishing apoptotic and non-apoptotic cell death in clinical sample including solid tumor, development of the method and utilization for clinical practice will be required in the future studies. Nevertheless, with respect to assessment of apoptotic cell death by TUNEL method, the clinical relevance has been reported in breast cancer patients who received neoadjuvant treatment with trastuzumab before chemotherapy. The tumor samples were collected by core needle biopsy after the treatment. Although the clinical response in the treatment with trastuzumab was associated with induction of apoptotic cell death, non-apoptotic cell death also needs to be assessed by standardized method in the tumor samples.

Clinically, it is hard to assess the relative contributions of apoptosis and non-apoptotic forms of cell death to therapeutic outcome. Since a number of recent preclinical studies suggest a significant contribution of non-apoptotic forms of cell death, including autophagy, necrosis, and senescence, as well as apoptosis, to overall tumor sensitivity, additional clinical studies are required to clarify the role of non-apoptotic forms of cell death in therapeutic outcome. Cellular damage induces growth arrest and tumor suppression by inducing various types of cell death, including apoptosis, necrosis,
autophagy, and senescence; the mechanism of cell death depends on the magnitude of DNA damage for heterogeneous populations of cancer cells (Fig. 4). Since induction of apoptosis likely has a limited role in determining the overall tumor response, there may be some benefit to targeting other forms of cell death in order to improve therapeutic outcomes and survival in cancer patients. Further studies are required to determine the relationship between various cell death pathways in order to limit tumor proliferation.

References


Do not hallucinate.