Balance between cell division and cell death is of utmost importance for the development and maintenance of multicellular organisms. Disorders of either process have pathologic consequences and can lead to disturbed embryogenesis, neurodegenerative diseases, or the development of cancer. Therefore, the equilibrium between life and death is tightly controlled and faulty elements can effectively be eliminated by a process called “programmed cell death” (PCD; ref. 1). In the past decades, PCD was held synonymous with apoptosis, a death process characterized by morphologic changes such as shrinkage of the cell, condensation of chromatin, and disintegration of the cell into small fragments (so-called “apoptotic bodies”) that can be removed by phagocytosis (2). The apoptotic cascade can be initiated via two major pathways, involving either the release of cytochrome c from the mitochondria (mitochondrion pathway), or activation of death receptors in response to ligand binding (death receptor pathway; ref. 3, 4). Upon triggering of either pathway, a specific family of cysteine proteases, the caspases, is activated to execute the cell’s fate in a programmed manner, leading to the typical morphologic changes (5). Whereas apoptosis is an inherent, controlled cellular death program, the conceptual counterpart, necrosis, is a more chaotic way of dying, which results from circumstances outside the cell, and is characterized by cellular edema and disruption of the plasma membrane, leading to release of the cellular components and inflammatory tissue response (2, 6).

In recent years, it has become evident that the classic dichotomy of apoptosis versus necrosis is a simplification of the highly sophisticated processes which guard the organism against unwanted and potentially harmful cells. Although caspases may be indispensable for the typical apoptotic morphology, the process of caspase activation is not the sole determinant of life and death decisions in PCD (7–11). One of the first clear demonstrations of caspase-independent PCD was given by Xiang et al., who showed that inhibition of caspase activities in the human leukemic cell line Jurkat did not inhibit Bax-induced cell death itself but only changed the apoptotic morphology of the dying cells (12). Indeed, more evidence is now accumulating that PCD can occur in complete absence of caspases, and other, noncaspase proteases have been described to be able to execute PCD (13–19). In addition, Cauwels et al. have shown that caspase inhibition did not alleviate but rather exacerbated tumor necrosis factor (TNF)-α–induced toxicity in mice, indicating that caspase-independent PCD is not restricted to in vitro models (20, 21). The various forms of caspase-independent cell death cannot readily be classified as “apoptosis” or “necrosis,” and alternative types of PCD have been described (7, 8, 10, 13, 22–24). They do not occur only under physiologic circumstances but can also be induced by for instance TNF-α or chemotherapeutic drugs (25). In this review, we focus on the various types of PCD and their death pathways at molecular and organelle level and discuss several stimuli that can lead to caspase-independent cell death.

**Classification of Programmed Cell Death**

The various types of PCD have in common that they are executed by active cellular processes that can be interpreted by interfering with intracellular signaling. This distinguishes them from accidental necrosis (22). Because it has become clear that inhibition of caspase activation does not necessarily protect against cell death stimuli but rather can reveal or even enhance...
underlying caspase-independent death programs, several models have been proposed (Fig. 1).

One model involves autophagy, which is also called type II cell death to distinguish it from apoptosis or type I cell death (23, 26). This process is characterized by sequestration of bulk cytoplasm and organelles in double or multimembrane autophagic vesicles and their delivery to and subsequent degradation by the cell’s own lysosomal system (autophagy is self-digestion in Greek). It serves to eliminate long-lived proteins and organelle components and has an important function in cellular remodeling due to differentiation, stress, or damage induced by cytokines. Cells that undergo excessive autophagy are triggered to die in a nonapoptotic manner, without activation of caspases (reviewed in ref. 27). Interestingly, autophagy may factor into both the promotion and prevention of cancer, and its role may be altered during tumor progression (reviewed in ref. 28). The autophagic capacity observed during experimental animal carcinogenesis has been shown decreased, indicating that breakdown of the autophagy process may contribute to the development of cancer (29-31). This is supported by recent reports on the autophagy gene Beclin 1, which show that heterogeneous disruption of this gene leads to increased tumorigenesis in mice (32, 33). However, cancer cells may need autophagy to survive nutrient-limiting and low-oxygen conditions and autophagy may protect cancer cells against ionizing radiation by removing damaged elements (34, 35). The precise role of cell death by autophagy in mammals is, therefore, not yet fully understood (36).

Paraptosis has recently been characterized by cytoplasmatic vacuolation that begins with progressive swelling of mitochondria and the endoplasmic reticulum (ER). It typically does not response to caspase inhibitors nor does it involve activation of caspases, the formation of apoptotic bodies, or other characteristics of apoptotic morphology (10). Paraptosis has been described to be mediated by mitogen-activated protein kinases (37) and can be triggered by the TNF receptor family member TRAIL/TROY (38) and the insulin-like growth factor I receptor (37).

...
Organelles Involved In Programmed Cell Death

Mitochondria. Release of toxic proteins form the intermembrane space of the mitochondria triggered by permeabilization of the outer mitochondrial membrane constitutes the “point of no return” in most cases of PCD (Fig. 3). Members of the Bcl-2 family control this process tightly (46): upon apoptotic signals, proapoptotic Bcl-2 proteins such as Bax and Bak are activated, resulting in outer mitochondrial membrane permeabilization. In contrast, antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-XL, can prevent this occurrence by heterodimerization with Bax-like proteins. Other proapoptotic Bcl-2 proteins which contain only the BH3 domain (e.g., Bad, Bid, Bim, Bmf, and Noxa) act by opposing the inhibitory effect of Bcl-2 or Bcl-XL, or by activating Bax-like proteins by direct binding. A second mechanism of permeabilization of the outer mitochondrial membrane is the opening of a permeability transition pore in the inner mitochondrial membrane upon a variety of stimuli. This allows water and small molecules (up to 1.5 kDa) to pass through, leading to swelling of the intermembrane space and rupture of the outer mitochondrial membrane (reviewed by Green (46)).

The first protein shown to be released from the mitochondria upon apoptotic stimuli is cytochrome c, an essential component of the respiratory chain. Upon release in the cytoplasm, it forms, in the presence of ATP, the so-called “apoptosome” together with Apaf-1 and caspase 9. This triggers the classic apoptotic cascade, leading to apoptotic cell death. The catalytic function of cytochrome c is safeguarded by members of the inhibitor of apoptosis proteins family, which are in turn controlled by two other mitochondrial proteins, Smac/DIABLO and OMI/HtrA2 (Fig. 3). In this way, OMI/HtrA2 plays a role in caspase-dependent cell death, but it can also act as an effector protein in necrosis-like PCD. This function is independent of its inhibitor of apoptosis proteins–binding activity but is done by its protease activity (47–50). It is, however, difficult to make firm conclusions about its precise contribution to cell death, as down-regulation of OMI/HtrA2 expression influences both its mitochondrial function and its cytosolic role in cell death (51). Another mitochondrial protein that potentially contributes to both caspase-independent and caspase-dependent cell death is endonuclease G. This protease is evolutionarily conserved with orthologues known in bacteria.
and fungi and is able to induce caspase-independent DNA fragmentation in isolated nuclei (52). It is likely that endonuclease G cooperates with caspase-activated exonucleases and DNase I to generate internucleosomal DNA fragments under physiologic conditions (53), and it remains to be established whether endonuclease G defines a single mitochondrial DNA fragmentation pathway in mammalian cells (25).

Apoptosis inducing factor (AIF) is a mitochondrial protein that plays a pivotal role in PCD, as shown by Joza et al., who reported that targeted disruption of the AIF gene inhibited the first wave of programmed cell death during embryogenesis (54). AIF was first described by Susin et al. (55) and is normally retained in the intermembrane mitochondrial space, where it performs an oxidoreductase function (56). Similar to the bifunctional role of cytochrome c, AIF becomes an active cell killer when it is released to the cytosol; it then translocates to the nucleus and triggers, possibly together with endonuclease G (57), peripheral chromatin condensation and high molecular weight (50 kb) DNA loss (58–60). The lethal effects of AIF are controlled by the antiapoptotic protein heat shock protein 70 that interacts with AIF and protects against its apoptogenic effects (61).

Interestingly, the lysosomal protease cathepsin D has been reported to trigger AIF release independent of the caspasecascade (62), and AIF mediated cell death in Apaf 1−/− and caspase 3−/− cells (63). In addition, the presence of the broad caspase inhibitor zVAD-fmk did not prevent the mitochondrial-nuclear translocation of AIF (64) nor did it prevent its lethal effects (58, 59), indicating that this protein is involved in caspase-independent, apoptosis-like PCD. This notion is supported by Yu et al., who showed that AIF release and subsequent cell death can be triggered independent of caspases by excessive calcium influx resulting in overactivation of poly (ADP-ribose) polymerase-1 (60). Furthermore, AIF and not caspase activation was shown largely responsible for pneumococcus-induced apoptosis in an experimental meningitis model (65), suggesting that caspase-independent cell death by AIF plays an important role in pathologic conditions. Others have, however, showed that mitochondrial release of AIF occurs downstream of cytochrome c in response to certain stimuli and may require caspase activation (66, 67). Apparently, AIF can serve as an additional response mechanism to facilitate the completion of caspase-dependent apoptosis in certain death paradigms, whereas it is capable of executing caspase-independent cell death in other cell types (reviewed in ref. 68). Indeed, there is now accumulating evidence in vitro as well as in vivo suggesting that AIF can act as a safeguard death executioner in cancer cells with faulty caspase activation (69–71).

Lysosomes. In the classic apoptosis-necrosis paradigm, lysosomes were solely considered involved in necrotic and autophagic cell death, and the lysosomal proteases were believed to take care only of nonspecific protein degradation within the lysosome. In recent years, however, it has become evident that the role of lysosomes in cell death is far more sophisticated. One of the first studies reporting an active role for lysosomal proteases in cell death was based on the cloning of "regression selected genes" in rat prostate and mammary glands after hormone ablation. Increased amounts of the lysosomal enzyme cathepsin B were found in the basal aspect of cells in regressing tissue, indicating that cathepsin B is required for the local degradation of the basement membrane, which is one of the earliest morphologically recognizable events of active cell death (72). Active participation of lysosomal proteases has since then been observed in cell death induced by several stimuli, including oxidative stress (73–77), TNF-α (16, 17, 78, 79), bile salt-induced apoptosis (80, 81), and chemotherapeutic drugs (15, 82).

Studies with the synthetic lysosomotropic detergent MSDH indicate that the key factor in determining the type of cell death is the magnitude of lysosomal permeabilization and the amount of proteolytic enzymes released into the cytosol (83). Whereas partial, selective permeabilization triggers apoptotic-like PCD, massive breakdown of lysosomes results in unregulated necrosis (reviewed in ref. 44). Several mechanisms to achieve the translocation of a balanced amount of lysosomal proteases to the cytoplasm, without risking a complete breakdown of the organelle and induction of necrotic cell death have been proposed. One theory involves accumulation of the lysosomotropic detergent sphingosine in the lysosomes, which could facilitate the release of lysosomal enzymes into the cytoplasm (18). Another possible mechanism is the generation of reactive oxygen species, which also can induce lysosomal leakage. Indeed, experimental evidence suggests that reactive oxygen species–induced lysosomal permeabilization usually precedes mitochondrial dysfunction (73, 74), thereby creating a feedback loop in which mitochondrial-reactive oxygen species can lead to more lysosomal permeabilization (84). An intriguing hypothesis is the translocation of proapoptotic members of the Bcl-2 family to the lysosomes, where they could induce the formation of pores and membrane permeabilization, similar to their well-known role in mitochondrial membrane polymerization (75–77). Recently, it has been described that heat shock protein 70, which antagonizes the apoptogenic effects of AIF, promotes cell survival by inhibiting lysosomal membrane permeabilization (85).

The cysteine protease cathepsin B and L and the aspartatic protease cathepsin D are the most abundant lysosomal proteases. Cathepsin B and D are most stable at physiologic, cytoplasmatic pH and seem to have the most prominent role in apoptotic and necrotic like PCD (reviewed in ref. 86, 87). Cathepsin B has been shown to translocate to the nucleus and thereby contribute to bile salt-induced apoptosis (81). Indeed, cathepsin B can act as an effector protease, downstream of caspases in certain cell types (16, 88), and is capable of executing cell death independent of the apoptotic machinery in WEHI-5 fibrosarcoma and non–small cell lung cancer (NSCLC) cells (15, 16). Other reports have, however, showed that lysosomal proteases rather promote cell death more indirectly by triggering mitochondrial dysfunction and subsequent release of mitochondrial proteins (14, 17, 82, 89, 90). This may occur via the Bcl-2 family protein Bid (19, 91, 92), which is cleaved and translocated to the mitochondria after lysosomal disruption by lysosomotropic agents (93). In addition, cathepsin D can trigger activation of Bax, leading to selective release of AIF from the mitochondria and PCD in T lymphocytes (62). Finally, lysosomal proteases have been reported to directly cleave and activate caspases, thereby confirming that lysosomal permeabilization often is an early event in the apoptotic cascade (94–96).

Taken together, it seems that lysosomal proteases trigger PCD not via a single specific pathway but rather via multiple pathways that may overlap with the traditional mediators of apoptosis (Fig. 3). The molecular identity of the mediators and
the necessity of activation of caspase-dependent pathways
remain to be elucidated in many cases and may vary depending
on the type of cells and the applied death stimulus (97). Many
other molecular pathways mediated by lysosomal enzymes are
likely to be described in the near future.

Endoplasmic reticulum. The ER is an important sensor of
cellular stress that can withhold protein synthesis and
metabolism to restore cellular homeostasis (98). If the damage
to the ER is too extensive, this can initiate PCD via the unfolded
protein response or via release of calcium into the cytoplasm
(reviewed in ref. 99). This leads to activation of caspase 12,
possibly via translocation of the Bcl-2 family member Bim to
the ER (100). Caspase 12 in its inactive state is localized at the
cytosolic face of the ER, but it triggers downstream caspases and
apoptosis when it becomes activated (101, 102). In addition and
independent of caspase 12 activation, ER stress can induce
permeabilization of the mitochondrial membrane and thus
activate the classic apoptotic pathway as well as other
mitochondrial death pathways (25, 103). Bcl-2 family proteins
as well as cytoplasmatic calcium shifts orchestrate the cross talk
between the mitochondria and the ER (104, 105).

In addition, intracellular calcium influx caused by ER stress
induces activation of a family of cytosolic proteases, the calpains
(calcium-activated neutral proteases), which normally reside in
the cytosol as inactive zymogens (106). Calpains have been
shown to act downstream of caspase activation and to
contribute to the degradation phase of camptothecin-induced
apoptosis in HL-60 cells (107, 108). They are kept in control by
their natural inhibitor calpastatin, which is in turn inactivated
by calpain- or caspase-mediated cleavage (109). In addition, Bax
and likely also other yet undefined pathways are involved in the
cross-talk between the calpain and caspase proteolytic system
(110, 111). Indeed, Sanvicens et al. have shown that both
caspases and calpains contribute to oxidative stress-induced
apoptosis in retinal photoreceptor cells (112). Furthermore, a
"calpain-cathepsin cascade" has been reported, in which
activated calpains induce release of lysosomal cathepsins and
subsequent cell death (Fig. 3; refs. 113, 114). Interestingly,
vitamin D compounds have been reported to trigger cell death
in MCF-7 cells executed by calpains in complete absence and
independent of caspase activation (115–117), indicating that
the ER may play a key role in certain types of caspase-
independent cell death (Fig. 3). This notion is supported by
several studies demonstrating an active and pivotal role for
calpains in anthracycline-induced toxicity in cardiac myocytes
(118), neuronal (119, 120), and pancreatic cell death (121).

Death Stimuli Triggering Alternative Types of
Programmed Cell Death

Caspase-dependent apoptosis plays a pivotal role in embryo-
genesis development, but there is now accumulating evidence
indicating that necrotic and apoptotic-like PCD are important
safeguard mechanisms for the developing organism (122). This
is illustrated by knockout studies in caspase 3 or 9/7−/− mice
which show, despite an altered morphology and temporal delay in
neuronal cell death, equal numbers of neurons that are ultimately
lost during development. Moreover, certain neurologic areas such
as the spinal cord and brainstem seem normal in both knockout
and control animals, suggesting that the involvement of specific
caspases and the occurrence of caspase-independent cell death
may depend on the brain region, cell type, or the death stimulus
(123). Similar results were found in the early motoneuron death
in the chick embryo cervical spinal cord, in which caspase activity
was involved but for which it was not indispensable (124). Many
other physiologic cell deaths do not seem to occur through
classic apoptosis but may primarily be executed by alternative
proteases (122). For instance, studies on maturation of osteo-
blasts in maturing bone (125), differentiation of keratinocytes
(126–128), and differentiation of lens fiber cells (94, 129, 130)
show that there is far more indirect than direct evidence that their
death is apoptotic or caspase dependent.

Triggering of the TNF receptor-1 (TNFR-1) by TNF-α can lead
to classic apoptosis via activation of the initiator protease
caspase 8 in the death receptor pathway (131). Other studies
have raised the possibility that TNF-α may trigger apoptosis via
an additional route, involving constituents of acidic vesicles
that can generate ceramide as a second messenger (132, 133).
Indeed, cathepsin D has been found to mediate PCD in HeLa
cells induced by TNF-α (134). In addition, cathepsin B has been
described to contribute to bile salt and TNF−α−induced
hepatocyte apoptosis (17, 79, 81, 135). The pivotal role of
cathepsin B in hepatocyte apoptosis has further been shown in
cathepsin B knockout mice, which were resistant to TNF-α−
mediated apoptosis (78). In addition, both genetic and
pharmacologic inhibition of cathepsin B reduces hepatic
inflammation and fibrogenesis upon bile duct ligation in mice
(80). Furthermore, it attenuates hepatocyte apoptosis and liver
damage in steatotic mice livers after cold ischemia/warm
reperfusion injury (136). These results suggest that cathepsin B
is indispensable for hepatocyte apoptosis induced by TNF-α
or liver injury and implicate that cathepsin B inhibition may be
of therapeutic interest in liver diseases (137).

Successful treatment with chemotherapeutic drugs is largely
dependent on their ability to trigger cell death in tumor cells
and activation of apoptosis is at least partially involved in this
process (138). The majority of cytotoxic agents trigger the
mitochondria pathway, but the death receptors have also been
reported to be involved in chemotherapeutic-induced apoptosis
(139, 140). However, recent evidence suggests that there are
forms of chemotherapy-induced cell death that cannot readily
be classified as apoptosis or necrosis but fit more in the
apoptosis-like/necrosis-like PCD model (22, 141, 142). Table 1
gives an overview of caspase-independent cell death induced by
chemotherapeutic agents. For instance, cell death induced by
paclitaxel and the novel microtubule-interacting agents epothi-
one B and discodermolide in NSCLC cells was not prevented
by the use of the broad-spectrum inhibitor zVAD-fmk nor was
it reduced in Bcl-2 overexpressing or Fas-associated death
domain-dominant negative cells, indicating that this class of
agents primarily induces caspase-independent cell death in
NSCLC cells (143, 144). Interestingly, specific cathepsin B
inhibitors, and not inhibitors of cathepsin D or calpains, did
reduce the lethal effects of these drugs, thereby providing
evidence for a cathepsin B−mediated cell death pathway
induced by microtubule stabilizing agents (15). Other studies
in NSCLC cells suggest that the relative resistance to caspase-
dependent apoptosis that is frequently seen in this cell type can
be circumvented by the triggering of an AIF-mediated, caspase-
independent mechanism (70) and AIF may determine the
chemoresistance of NSCLC cells (145). In addition, paclitaxel
induced caspase-independent apoptosis via AIF in ovarian
carcinoma cells (146), indicating that the activation of a certain death pathway may vary upon the cellular system (97). Although cell death in hematologic malignancies is more often mediated by the classic apoptotic proteases than in solid tumors (7, 141), the occurrence of caspase-independent cell death has been reported in T lymphocytes and acute myeloid leukemia (147, 148). Taken together, the cellular death response triggered by cytotoxic agents depends on the type and dose of chemotherapeutic stress within the cellular context and may involve classic apoptosis, as well as various types of apoptotic or necrotic PCD.

Conclusions

Despite the enormous importance of the discovery of apoptosis as a cell death program indispensable for embryogenesis and protection against unbridled cell growth, the apoptosis-necrosis paradigm is too simple to encompass the wide spectrum of possibilities we have to examine and potentially harmful cells. Not only caspases, but also calpains, endonucleases, and other proteases can execute programmed cell death, and they can be directed by several cellular organelles, including mitochondria, lysosomes, and the ER, which can act independently, or collaborate with each other. Although several models of caspase-independent cell death have been described, the various death routes may overlap and several characteristics may be displayed at the same time. The evolutionary advantage of the existence of multiple death pathways is obvious: it protects the organism against the development of malignant diseases as many burdens have to be overcome before a cell becomes a tumor cell. This may explain the relative rarity of cancer, in respect to the huge number of cell divisions and mutations during a human life. The growing knowledge of caspase-independent cell death pathways is important for the oncology field, as they could potentially be manipulated to develop new cancer therapies.

Table 1. Overview of caspase-independent cell death triggered by cytotoxic agents

<table>
<thead>
<tr>
<th>Cytotoxic agent</th>
<th>System</th>
<th>Caspase-independent cell death mediated by</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camptothecin</td>
<td>Hepatocytes</td>
<td>Cathepsin D</td>
<td>(135)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Human leukemic cells</td>
<td>AIF</td>
<td>(149, 150)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Acute myeloid leukemia cells</td>
<td>Not identified</td>
<td>(147)</td>
</tr>
<tr>
<td>Neuroblastoma [α-type]cells</td>
<td></td>
<td>Not identified</td>
<td>(151)</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td></td>
<td>Calpains</td>
<td>(118)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>NSCLC cells</td>
<td>Cathepsin B</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td>ovarian carcinoma cells</td>
<td>AIF</td>
<td>(146)</td>
</tr>
<tr>
<td>Arsenic trioxide</td>
<td>Myeloma cells</td>
<td>Not identified</td>
<td>(152)</td>
</tr>
<tr>
<td>T cell lymphoma cells</td>
<td></td>
<td>Not identified</td>
<td>(153)</td>
</tr>
<tr>
<td>Stauroporine</td>
<td>NSCLC cells</td>
<td>AIF</td>
<td>(145)</td>
</tr>
<tr>
<td></td>
<td>fibroblasts</td>
<td>Cathepsin D</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td>T lymphocytes</td>
<td>Cathepsin D, AIF</td>
<td>(62)</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>Gliona cells</td>
<td>AIF</td>
<td>(69)</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>breast cancer cells</td>
<td>Calpains</td>
<td>(115, 116)</td>
</tr>
<tr>
<td>Quinolone antibiotics</td>
<td>several human and mice cells</td>
<td>Cathepsin B and D, followed by mitochondrial permeabilization</td>
<td>(14)</td>
</tr>
</tbody>
</table>

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Cell Death Independent of Caspases: A Review

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