Targeting the Apoptosome for Cancer Therapy
Elizabeth C. Ledgerwood and Ian M. Morison

Abstract
Apoptosis is a programmed mechanism of cell death that ensures normal development and tissue homeostasis in metazoans. Avoidance of apoptosis is an important contributor to the survival of tumor cells, and the ability to specifically trigger tumor cell apoptosis is a major goal in cancer treatment. In vertebrates, numerous stress signals engage the intrinsic apoptosis pathway to induce the release of cytochrome c from mitochondria. Cytochrome c binds to apoptosis protease activating factor-1, triggering formation of the apoptosome, a multisubunit protein complex that serves as a platform for caspase activation. In this review we summarize the mechanisms of apoptosome assembly and activation, and our current understanding of the regulation of these processes. We detail the evidence that loss-of-function of the apoptosome pathway may contribute to the development of specific cancers. Finally we discuss recent results showing enhanced sensitivity of some tumor cells to cytochrome c–induced apoptosis, suggesting that agents able to directly or indirectly trigger apoptosome-catalyzed caspase activation in tumor cells could provide new approaches to cancer treatment.

Background
Apoptosis is a programmed mechanism of cell death that is essential for normal development and tissue homeostasis (1). Because dysregulation of apoptosis is a major contributor to the survival of tumor cells, there is much interest in identifying new ways to activate this process in malignant cells. The aim of the apoptotic program is to activate caspases, the family of proteases that drive the biochemical events leading to the organized disassembly of the cell. Two well-described pathways lead to caspase activation in mammalian cells: the extrinsic pathway, triggered by an extracellular signal, such as the binding of tumor necrosis factor or Fas ligand to cell surface receptors, and the intrinsic pathway that responds to intracellular signals such as DNA damage (1, 2).

The intrinsic apoptosis pathway is also referred to as the mitochondrial apoptosis pathway because it requires the release of proapoptotic factors from the mitochondrial intermembrane space, a key player being cytochrome c (2, 3). The discovery in 1996 that this electron carrier also acts as a cytosolic apoptosis effector (4) was described at the time as "bizarre and unexpected" (5). It is now known that multiple pathways activated by intracellular stress signals converge at the mitochondrial outer membrane. A key step in the process is the release of cytochrome c into the cytosol where it can bind to the apoptosis protease activating factor-1 (Apaf-1). This promotes assembly of the apoptosome, a protein complex that serves as a platform for activation of the initiator caspase procaspase-9. Subsequently procaspase-9 cleaves and activates executioner caspases such as caspase-3, leading to death of the cell. Cytochrome c, Apaf-1, and procaspase-9 form the core of the mitochondria-mediated intrinsic apoptosis pathway (refs. 7–9; Fig. 1).

Cytosolic cytochrome c is the major inducer of caspase activation downstream of mitochondria. The proapoptotic activity of cytochrome c requires interaction with apoptosis protease activating factor-1 (Apaf-1). This promotes assembly of the apoptosome, a protein complex that serves as a platform for activation of the initiator caspase procaspase-9. Subsequently caspase-9 cleaves and activates executioner caspases such as caspase-3, leading to death of the cell. Cytochrome c, Apaf-1, and procaspase-9 form the core of the mitochondria-mediated intrinsic apoptosis pathway (refs. 7–9; Fig. 1).

Manipulation of apoptosome assembly and activation has significant therapeutic potential, but relies on detailed understanding of the molecules involved in its formation and regulation. The crucial initiation step for apoptosome assembly is the binding of cytochrome c to the functional Apaf-1 splice variant, Apaf-1XL (10). Apaf-1 has three functional regions: the NH2-terminal caspase recruitment domain that binds procaspase-9, the central nucleotide-binding and oligomerization domain (NOD, also called the NB-ARC or Ced-4 domain), and 13 WD40 repeats in the COOH-terminal half. A 12.8 Å cryo-electron microscopy apoptosome structure shows that a single molecule of cytochrome c binds between two β-propellers formed by the WD40 repeats (11). This interaction is of high affinity and primarily electrostatic with contributions from lysine residues distributed over the entire surface of cytochrome c (12–16). Our recent report of mutation of glycine 41 to serine in human cytochrome c that increases its
caspase-activating activity (17) shows that the electrostatic interactions may not be the sole binding determinant. Cytochrome c undergoes conformational fluctuations, particularly in the region of residue 44, and it may be that there is a favored conformational state necessary for its binding to Apaf-1 (18, 19). In healthy cells Apaf-1 exists as an inactive dATP/ATP-bound monomer in which the WD40 domains are folded back on the rest of the protein, preventing oligomerization and caspase-9 binding (20, 21). The binding of cytochrome c induces a conformational change in Apaf-1, exposing the NH2-terminal regions and triggering hydrolysis of the Apaf-1–bound dATP/ATP. However, dADP-bound Apaf-1 remains inactive and only assembles into the apoptosome once the exchange of dATP for dADP has occurred (11, 21). Seven cytochrome c–bound Apaf-1 molecules assemble into a wheel-like complex with the caspase recruitment domains at the hub and the WD40–cytochrome c bound regions forming the spikes of the wheel (11). In this complex the Apaf-1 caspase recruitment domains are free to bind procaspase-9, which is then activated.

The cell has a number of mechanisms in place to ensure that active apoptosomes are only formed in response to apoptotic stimuli. Apoptosome assembly is inhibited by physiologic concentrations of potassium (22–25), calcium (26), and nucleotides (16, 25). High ionic strength disrupts the interaction between cytochrome c and Apaf-1, calcium binds to Apaf-1 preventing nucleotide exchange, and ATP binds to surface lysine residues on cytochrome c preventing binding to Apaf-1. During apoptosis the inhibitory effects are reduced as the concentration of cytochrome c increases and the cytosolic concentrations of these factors decrease (16, 27, 28). This combination of mechanisms ensures that apoptosome formation does not occur in response to “accidental” release of small amounts of cytochrome c in healthy cells.

Beyond these factors, our understanding of how the apoptosome is regulated in normal cells and in cancer cells is in its infancy. In Caenorhabditis elegans, the Bcl-2 homologue CED-9 binds directly to the Apaf-1 homologue CED-4 to inhibit caspase activation. It was therefore expected that apoptosome assembly and activity would be regulated by additional protein factors. Early reports suggested that Bcl-XL plays a similar role in vertebrates as CED-3 does in C. elegans, binding to Apaf-1 to regulate its activity (29, 30). However, subsequent results have not sustained these observations (31).

The isolation of endogenous active apoptosomes from cultured cells has enabled characterization of the components by mass spectrometry (32, 33). Only Apaf-1, caspase-9, caspase-3, and X-linked inhibitor of apoptosis protein (XIAP) are present. XIAP is a caspase-9 inhibitor, which in healthy cells contributes to the prevention of accidental caspase activation. During apoptosis, the presence of XIAP in the apoptosome is initially required for binding of caspase-3 (33). Subsequently, the proapoptotic factors Smac/Diablo and Omi/Htra2, which are released from mitochondria alongside cytochrome c, bind to and displace XIAP from the apoptosome (7, 32). Interestingly, cytochrome c was only transiently associated with the isolated apoptosomes, suggesting it is necessary for apoptosome assembly but not for apoptosome activity. The failure to detect additional proteins in the apoptosome complex in these studies does not, however, rule out regulatory roles for other proteins. Indeed a large number of proteins have been reported to enhance or inhibit apoptosome assembly and/or activity, although only a few of these effects have been independently confirmed. For example, it was initially reported that heat

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**Fig. 1.** Cytochrome c–induced apoptosome formation and caspase activation. In healthy cells cytochrome c is sequestered in the mitochondrial intermembrane space and Apaf-1 exists as an inactive monomer in the cytosol. In response to stress stimuli (e.g. DNA damage or chemotherapeutic drugs) cytochrome c is released from mitochondria to the cytosol where it binds to Apaf-1, triggering a conformational change and hydrolysis of the Apaf-1–bound dATP/ATP. In a process dependent on exchange of dATP/ATP for dADP/ADP, the Apaf-1–cytochrome c heterodimers assemble into the apoptosome, which provides a platform for activation of the initiator caspase procaspase-9. Activated caspase-9 then cleaves and activates executioner caspases such as caspase-3.
shock protein (Hsp) 70 inhibited apoptosome-dependent caspase activation (34, 35); the inhibitory effect of Hsp70, however, has subsequently been shown to be due to high salt concentrations in Hsp70 preparations (32, 36). Similarly, Hsp27 and Hsp90 have been reported to bind to cytochrome c (37) and Apaf-1 (38, 39), respectively, thereby inhibiting apoptosome activity, although for Hsp27 this result has been disputed (35).

There is accumulating evidence that pp32/putative HLA class II-associated protein-1 (PHAP1) is a bone fide apoptosome activator. pp32/PHAP1 indirectly increased both procaspase-9 association with the apoptosome and caspase-9 activity, without altering apoptosome assembly (40). A recent report suggests PHAP1 requires at least Hsp70 and cellular apoptosis sensitivity protein as Apaf-1-binding cofactors to promote nucleotide exchange on Apaf-1, thereby enhancing apoptosome formation (41). Prothymosin-α is a putative negative regulator of the apoptosome pathway. Prothymosin-α blocks the stimulatory activity of PHAP1 (40), and knockdown of prothymosin-α expression sensitizes HeLa cells to UV and staurosporine-induced apoptosis (40, 42). One report suggested prothymosin-α interacts with p8 to inhibit cytochrome c–induced caspase activation (42); however, prothymosin-α also binds directly to cytochrome c (43), providing an additional mechanism for inhibition.

Other proteins reported to regulate apoptosome formation and/or activation include histone H1.2, HCA66, NAC/CARD7, Nuclung, AFP, AChE (enhancers), and Bcr-Abl, Aven, AIP, APIP, JNK1&2, PKA, ERK, Akt, PKCζ, HBXIP, and TUCAN (inhibitors; reviewed in ref. 44). In addition, modification of cytochrome c itself may alter its ability to bind to Apaf-1 and induce apoptosis (45, 46). The involvement of many of these proteins in multiple cellular processes and the disparity between findings from different laboratories and in different cell types make it difficult to determine physiologic relevance.

**Clinical-Translational Advances**

Because the inhibition of apoptosis is one of the key features of cancer (47), identifying ways of overcoming or bypassing this inhibition to activate caspases has become an important strategy in the development of new cancer treatments. To date major efforts to achieve this have involved targeting the death receptors, Bcl-2 family proteins, inhibitors of apoptosis, and direct caspase activation (48). In contrast, activation of the apoptosome has received relatively little attention (44, 49, 50). However, characterization of the apoptosome pathway in specific cancers suggests it is a valid target. In particular, in tumors that are refractory to treatments aimed upstream of mitochondrial cytochrome c release, but which have intact postmitochondrial apoptotic machinery (51), the ability to directly cause apoptosis activation could be valuable. The profiling of a number of cancer and normal cell types has been undertaken to determine when the apoptosome would be a valid target and whether it is possible to capitalize on differences between tumor and normal cells (reviewed in refs. 44, 51, 52).

Melanoma, which is generally refractory to chemotherapy, was the first cancer in which loss of Apaf-1 was shown (53–55). However, others have not been able to replicate these findings in melanoma, and have suggested that difficulties in detecting Apaf-1 protein may lead to incorrect conclusion of Apaf-1 loss (56, 57). Loss of Apaf-1 has also been reported in ovarian and leukemic cancer cell lines, and in glioblastomas (58–60), and resistance to cytochrome c–induced caspase activation was common in cells isolated from patients with acute myeloid leukemia (61). Sequestration of Apaf-1 away from the cytosol has been proposed as an alternative mechanism of apoptosis resistance in B-cell lymphoma (62). Analysis of cytochrome c–induced apoptosis in a panel of non–small cell lung cancer cell lines found that primary drug resistance correlated with an inability to activate caspases-9 and caspase-3 although apoptosome formation was normal (63).

This inhibition was overcome by the addition of pp32/PHAP1 both in the cell lines and in a murine tumor model in vivo, but because there was no difference in pp32/PHAP1 levels in the resistant versus sensitive cell lines, the mechanism of resistance is unclear.

Investigation of cells isolated from different stages of colon cancer showed that the cells became more resistant to treatment with cisplatin, ionizing radiation, or Fas ligation as the disease progressed. Resistance correlated with the acquisition of genetic changes in apoptosis effectors, including down-regulation of Apaf-1 and up-regulation of XIAP (64). However, when the ability of cytochrome c to induce caspase activation was determined in cytosols prepared from a range of lung, colon, stomach, brain, and breast cancer cell lines, most cells were found to be more sensitive to cytochrome c–induced caspase activation than normal cells (65). Breast tumors are significantly more sensitive to microinjected cytochrome c than surrounding normal mammary epithelial cells (66). This difference has been attributed to the increased levels of pp32/PHAP1 in the cancer cells. Similarly, whereas normal brain tissue is highly resistant to cytochrome c–induced apoptosis due to low expression of Apaf-1, brain tumors have increased expression of Apaf-1 (67). This results in increased susceptibility of human glioblastoma and medulloblastoma cell lines, and mouse high-grade astrocytomas and medulloblastomas, to apoptosis following cytosolic microinjection of cytochrome c.

High levels of Apaf1 have previously been associated with the inability of endogenous inhibitors of apoptosis to protect pheochromocytoma cells against cytochrome c–mediated apoptosis (68).

Cell-free apoptosis assays have been used to screen small molecule libraries for compounds that induce apoptosome activation (40, 69). α-(Trichloromethyl)-4-pyridineethanol (PETCM), which was identified in a high-throughput screen, enhanced cytochrome c–induced caspase activation in vitro in the absence of added nucleotides, and could overcome the inhibitory effect of prothymosin-α (40). A series of indolene and carbamate compounds also induced caspase activation by an unknown mechanism, and one of these (compound 2) had proapoptotic activity in cell lines (69). The activity of PETCM and compound 2 required the presence of cytochrome c.

One approach that is currently being explored is the development of small molecule cytochrome c mimetics, which will trigger the conformational change in Apaf-1 that is necessary for apoptosome assembly and caspase-9 activation (66, 67). Another way of targeting the apoptosome for cancer treatment is the use of compounds that directly induce cytochrome c release from mitochondria. Triacsin c, an inhibitor of acyl-CoA-synthase, selectively induced cytochrome c...
c release and apoptosome-mediated cell death in tumor cells and decreased tumor growth in a xenograft model (65). In cancers where a decrease in apoptosome components contributes to drug resistance, it may prove beneficial to use agents that increase expression of apoptosome components. For example the ability of the pan–histone deacetylase inhibitor LAQ824 to cause cell death is associated with induction of Apaf-1 and procaspase-9 expression (70).

In recent years significant progress has been made in understanding the core components of the apoptosome, but there are still many questions regarding its regulation. Defects in the apoptosome pathway may contribute to the development of some cancers and to chemoresistance. Based on our emerging knowledge, however, being able to trigger cell death via the apoptosome may provide a means to specifically kill tumor cells in some malignancies. The ongoing study of the apoptosome will inform the development of drugs that can activate the apoptosome and thus provide new potential targets for drug design.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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