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News and Commentary

Proteases, proteasomes and apoptosis: breaking Ub is hard to do

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Studies over the past decade have firmly established that apoptosis is essentially the end product of the controlled activation of members of the caspase family of cysteine proteases.[1] While there is still much debate concerning the precise routes to activation of initiator caspases in response to specific death stimuli, it is nonetheless widely accepted that caspase activity is required for the appearance of most of the defining features of apoptosis. However, we are still a long way from fully understanding how caspases coordinate apoptosis, as although hundreds of substrates for these proteases have been identified, the consequences of most of these proteolytic events still remains unclear.[2]

Other proteases have also consistently featured in the cell death literature over the past decade. Cytotoxic lymphocyte-derived granzymes are specialized lysosomal proteases that are delivered to target cells during CTL/NK killing and can initiate apoptosis in the target through activation of caspases.[3] CTL/NK granule proteases can also contribute to cell death by other mechanisms.[4] The cathepsin proteases, which are close relatives of the granzymes, have also been implicated in cell killing in response to cytotoxic drugs that provoke lysosomal rupture.[5] Accumulating evidence also suggests that the multicatalytic proteasome protease complex may play an important role in setting a threshold for apoptosis through controlling the degradation of key triggers of the cell death machinery. Owing to the special nature of this issue of *Cell Death and Differentiation*, here we will focus on some of the major unresolved issues concerning the role of proteases in apoptosis with particular reference to the role of the ubiquitin (Ub)-proteasome system and its influence on cell death.

Caspases: till death do us participate

In general, caspases are essentially dormant in healthy cells and become activated during apoptosis through separation of their large and small subunits by autocatalytic means or through proteolysis by other caspases.[6] Several caspase activation complexes have been identified to date and several others have been proposed but remain poorly defined or controversial at present.

Two major pathways to initiator caspase activation have been firmly established thus far (Figure 1). In the extrinsic route to caspase activation, members of the death receptor family (such as CD95/Apo-1/Fas or TNFR) promote activation of caspase-8 via the adaptor molecule FADD.[7] The intrinsic or apoptosome route is initiated by diverse stimuli that converge on mitochondria and promote mitochondrial outer membrane permeabilization (MOMP) followed by the release of mitochondrial proteins from the intermembrane space, such as cytochrome *c*. Upon release into the cytosol, cytochrome *c* promotes association between caspase-9 and its adaptor protein, Apaf-1, which results in activation of the former and the propagation of a cascade of further caspase activation events as a consequence. The latter pathways have been extensively reviewed elsewhere[7,8] and will not be discussed in detail here. Other routes to apoptosis-associated caspase activation have been proposed but these remain relatively obscure, at least in molecular terms, to date.

Perhaps the most hotly debated route to caspase activation is the endoplasmic reticulum (ER) stress-associated pathway. Initial observations using *casp-12* null mice suggested that cells from these animals were relatively refactory to stimuli that provoke ER stress, such as thapsigargin and tunicamycin.[9] However, the nature of the hypothetical complex that is responsible for caspase-12 activation during ER stress remains elusive. Furthermore, it is now clear that few, if any, humans express a functional caspase-12 enzyme but do respond to triggers of ER stress by undergoing apoptosis.[10] Murine caspase-12 is a highly unusual caspase that fails to display proteolytic activity towards the normal range of substrates that caspases typically hydrolyse ([11] and Logue and Martin, unpublished observations). It has been suggested that human caspase-4 may functionally substitute for the absence of a functional caspase-12 gene in man.[12] However, human caspase-4 does not display an activity profile similar to murine caspase-12 and the sequence identity of this caspase, as well as its chromosomal location, groups caspase-4 with the inflammatory caspases. Therefore, the proposal that human caspase-4 is the functional homologue of murine caspase-12 remains speculative.

In the absence of a functional caspase-12 gene in man, it seems very unlikely that this caspase sits at the apex of the ER stress-initiated cell death pathway. Interestingly, recent studies suggest that murine caspase-12 may be proteolytically processed downstream of the Apaf-1 apoptosome.[13] Using Bax/Bak-null or caspase-9-null MEFs, Ruiz-Vela and co-workers have shown that caspase-12 failed to be processed in response to brefeldin A, a classical trigger of the ER stress pathway. The latter observation argues that processing of caspase-12 is a late rather than an early event during ER stress-associated apoptosis. Moreover, these observations also suggest that triggers of ER stress may kill via the apoptosome-driven caspase activation cascade.

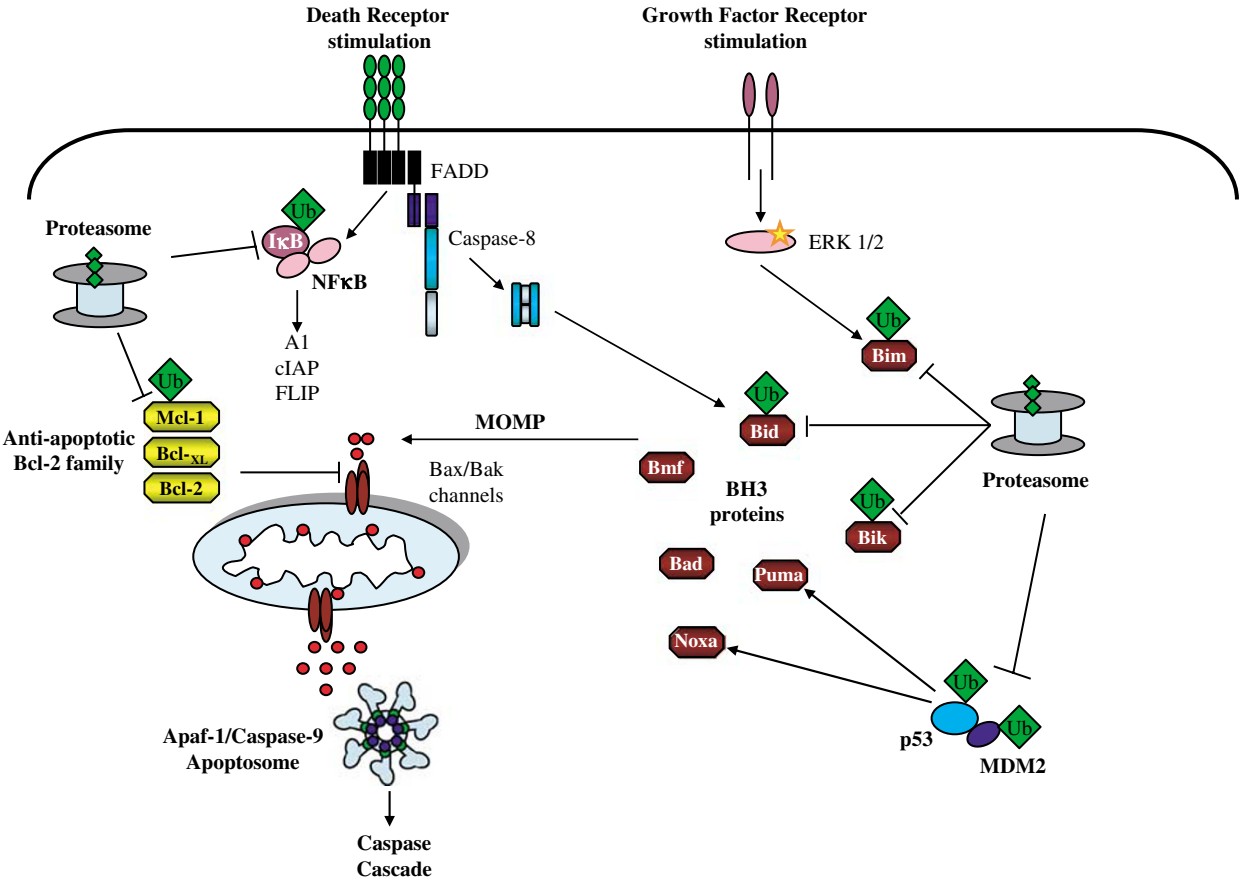

Figure 1 Modulation of cell death pathways by the Ub-proteasome system. Caspase activation during apoptosis can be achieved in two major ways. In some contexts, external ligation of death receptors leads to activation of caspase-8, mediated by the adapter protein, FADD. Alternatively, the intrinsic/apoptosome pathway is activated by stimuli that mobilize BH-3-only proteins, causing Bax/Bak oligomerization and MOMP. This causes cytochrome *c* release from mitochondria, triggering Apaf-1 oligomerization, caspase-9 activation and induction of the caspase cascade. In some contexts of death receptor signalling, caspase-8 cleaves BH-3-only protein Bid, thereby accessing the apoptosome pathway as described above. The Ub-proteasome pathway may block cytochrome *c* release by targeting several cell death regulators. The BH-3-only proteins Bim, Bik and caspase-cleaved Bid (tBid) undergo ubiquitination and proteasome-mediated degradation. The Bcl-2-related protein, Mcl-l, has a short protein half-life and is subject to proteasome-mediated degradation. The proteasome plays a major role in promotion and repression of p53-induced apoptosis by mediating degradation of both p53 as well as its E3 ligase, Mdm2. In addition to repressing cell death, proteasome-mediated degradation of IκB facilitates transcription of NFκB target genes, thereby promoting cellular survival

Lively debate has also surrounded the proposal that caspase-2 plays an initiating role in certain contexts associated with DNA damage.[14] While caspase-2 has all of the structural features of an initiator caspase, there is little functional evidence that this protein can act in this capacity. Studies using siRNAs to silence caspase-2 expression provided the initial basis for suggestions that caspase-2 may act upstream of mitochondria and be required for mitochondrial cytochrome *c* release in response to certain death stimuli normally associated with the apoptosome pathway to apoptosis.[14] However, subsequent investigations by the original authors of this study, as well as by other investigators, now suggest that these effects are likely to have been due to nonspecific 'off-target' effects of the siRNAs used in the original study.[13,14] Off-target effects of siRNA are much more common than was initially appreciated and several recent studies have shown that supposedly specific siRNAs can often silence cohorts of genes rather than the intended target.[15] It is also worth recalling that caspase-2-null mice exhibit essentially normal phenotypes and respond normally to diverse death stimuli.[16] This is unlikely to be due to functional redundancy between caspase-2 and any other caspase because caspase-2 also exhibits a somewhat unusual activity profile (it fails to process any other caspase for instance) and fails to cleave proteins that many other cell death-associated caspases do.

Opening channels of communication to the apoptosome

Much evidence now suggests that a major mechanism for caspase activation in apoptosis is the release of mitochondrial cytochrome *c* from the mitochondrial intermembrane space. Cytochrome *c* acts in a 'hit and run' manner to trigger assembly of the Apaf-1/caspase-9 apoptosome[17] and this complex drives several additional caspase activation events to disseminate the caspase activation cascade downstream. It is becoming increasingly clear that MOMP is a point of no return in many pathways to apoptosis and interventions aimed

at rescuing cells after MOMP may be pointless. This appears to be largely due to the release of multiple intermembrane space proteins, including cytochrome *c*, during MOMP and the subsequent death of cells irrespective of whether caspase activation is blocked downstream or not. This phenomenon is often called 'caspase-independent cell death' although this mode of cell death may be a purely *in vitro* phenomenon due to artificial disablement of the caspase activation cascade. In real biological contexts, MOMP will almost always lead to robust caspase activation via activation of the Apaf-1 apoptosome. Nonetheless, from studies using cells lacking Apaf-1 or caspase-9, as well as other lines of investigation, it is readily apparent that most cell types are doomed upon MOMP irrespective of whether caspase activation can occur downstream or not.[18] It is important to point out that caspase-independent cell death, resulting from MOMP in the context of caspase inhibition, does not exhibit the typical hallmarks of apoptosis (such as plasma membrane blebbing, apoptotic body production, internucleosomal DNA fragmentation and phosphatidylserine externalization in the absence of vital dye uptake), but cells die irrespective of the absence of these features.

Elegant work performed by Stanley Korsmeyer and co-workers has established that the Bax and Bak proteins are central to MOMP in the context of numerous cell death-initiating stimuli.[19] Upon activation, Bax and Bak are capable of forming channels that permeabilize the mitochondrial outer membrane and permit the escape of numerous intermembrane space proteins during apotosis, including cytochrome *c*.[20] In healthy cells, MOMP is prevented through the actions of Bcl-2 and its close relatives such as Bcl-xL and Mcl-1 that oppose the actions of Bax and Bak. Death-inducing stimuli that trigger MOMP appear to do so by activating one or more members of the BH3-only protein family that promote opening of the Bax/Bak channel (Figure 1). Recent papers suggest that BH3-only proteins come in two main flavours; those that directly open the Bax/Bak channel (such as Bim and Bid) and those that lower the threshold for channel opening through neutralizing Bcl-2 and its functional relatives.[21,22] Through a series of definitive gene-targeting studies in mice from Strasser and co-workers, it has been established that BH3-only proteins play key decision-making roles in apoptosis.[23–25] The BH3-only proteins appear to act as pathway-specific sentinels for cell stress, damage, starvation, loss of matrix attachment, CTL attack (via granzyme B) and death receptor signalling that couple these signals for apoptosis to the Bax/Bak channel.[23] Thus, regulation of the availability of these proteins within the cell plays an important role in setting a threshold for apoptosis. It is in this context where the Ub-proteasome pathway may exert a major influence on the cell death machinery.

The Ub-proteasome system and apoptosis

The Ub-proteasome system clearly exerts multiple layers of influence over the daily lives of cells at numerous points. Loss of virtually any of the multiple core subunits of the 20S proteasome results in loss of cell viability,[26] a testament to the essential role of this protease complex in normal cellular function. Interestingly, recent reports also suggest that proteasome function may be inhibited during apoptosis through targeting of several proteasome subunits for caspase-dependent proteolysis.[27,28] However, proteasomes have also been implicated in the regulation of apoptosis in more specific ways. The inhibitor of apoptosis proteins (IAPs) are a family of caspase inhibitors that can delay apoptosis upon overexpression in a variety of cell types. Several IAPs can also act as Ub ligases as these proteins contain RING domains and can promote polyubiquitination of their substrates.[29] However, although the IAPs that display Ub ligase activity can promote their own destruction through auto-ubiquitination, little evidence exists to suggest that they act as Ub-ligases towards their caspase-binding partners. Thus, although it is tempting to speculate that the Ub-ligase activities of the IAPs can regulate the destruction of caspases by targeting active caspases to the proteasome, the IAPs seem to be conspicuously poor Ub-ligases for caspases. In any case, inhibition of caspase activity via Ub-mediated turnover is unlikely to prevent cell death, because in many instances these events occur downstream of MOMP. However, where the Ub-proteasome system may really come into play may be at the level of the BH3-only proteins discussed above. Given that the BH-3-only proteins operate upstream of MOMP, Ub-proteasome intervention at this point has the potential to determine whether a cell will live or die. Indeed several reports now suggest that the availability of Bim, Bid and Bik are influenced by the ongoing destruction of these proteins by the Ub-proteasome pathway.[30,31] Stimuli that promote destabilization of BH3-only proteins by targeting these proteins for polyubiquitination and proteasome-mediated destruction may, therefore, play important roles in regulating thresholds for apoptosis.

Inhibition of the proteasome may also contribute to the initiation of apoptosis in several other ways. The NFκB pathway, an important determinant of cell survival, is regulated by proteasome-mediated degradation of the NFκB inhibitor IκB. This releases NFκB to activate numerous transcriptional targets including cell death inhibitors such as Bcl-2, A1, cIAP1 and FLIP.[32] Suppression of NFκB activation dramatically increases the susceptibility of cells to TNF and DNA-damaging drugs.[33] The p53 tumour suppressor pathway is also regulated by the proteasome as p53 is kept at constitutively low levels by proteasome-mediated degradation following ubiquitination by its E3 ligase, Mdm2. Stabilization of p53 as a consequence of DNA damage can then lead to cell cycle arrest and apoptosis. With such a range of functions, it is perhaps unsurprising that the inhibition of proteasome function should be lethal to cells. Apoptosis resulting from inhibition of the proteasome is typically accompanied by the stabilization of BH3-only proteins including Bik and Bim, suggesting this as a principal mechanism by which the proteasome regulates cell survival.[34]

Targeting the proteasome for cancer therapy: the benefits of breaking Ub

Since inhibition of proteasome function has revealed itself to be such a potent cell killing stimulus, attention has turned in

recent years towards the use of proteasome inhibitors as anticancer drugs.[35] Since all cells require proteasome activity, it might seem that such inhibitors would cause unacceptable collateral damage to nontransformed tissues. However, some tumours exhibit higher rates of proteasome activity and seem to be more susceptible to proteasome inhibitors than their nontransformed counterparts.[36] This is possibly because tumour cells typically proliferate and grow at much faster rates than nontransformed cells, thereby generating a heavier demand for proteasome activity in tumours. Also, the presence of genetic defects in neoplastic cells may cause the accumulation of mutant proteins in the cell, which could well prove toxic without high levels of proteosome activity to remove such proteins.

In addition to the above, it is likely that the specific toxicity of proteasome inhibitors towards neoplastic cells relies heavily upon inhibition of prosurvival pathways and unlocking death pathways typically dysregulated during transformation. Proteasome inhibition seems to cause arrest of cells at the G2/M boundary, overriding the loss of cell cycle controls typically acquired during transformation. Another control pathway frequently bypassed in many tumours is the p53-dependent pathway to apoptosis, as many tumours carry inactivating mutations in p53. Additionally, other tumours display increased levels of MDM-2, causing constitutive ubiquitination and degradation of p53. Proteasome inhibition in this context might stabilize p53 and reactivate the disabled checkpoint, leading to cell death.

BH3-only proteins, as discussed above, are key mediators of apoptosis that can be regulated by proteasome-mediated degradation, and it appears that some cancer cell lines may enhance survival by maintaining very low levels of proteins such as Bim.[37] By stabilizing these death regulators, proteasome inhibitors may resensitize cells to apoptotic stimuli. Inhibition of NFκB-driven prosurvival pathways by the expression of a constitutively active, nondegradable, IκB can strongly sensitize transformed cells to death stimuli.[33] Proteasome inhibition may have the same effect by preventing the degradation of IκB. Other effects of proteasome inhibitors may not only target the tumour but may also target cells of the microvasculature that supply it, as discussed later. Vascular endothelial cells may be vulnerable to proteasome inhibition as a result of their high rates of proliferation.

So, does the theory meet with reality in the clinical setting? One proteasome inhibitor currently under development as an antineoplastic agent is the boronic acid dipeptide Bortezomib.[35] This drug was isolated in a screen of boron-containing compounds against the National Cancer Institute (NCI) panel of 60 cancer cell lines. Boron-containing compounds have a strong and selective inhibitory activity against the chymotrypsin-like site of the 20S proteasome, and Bortezomib potently induced death in a broad range of cancer cell types. The drug is now in Phase II clinical trials and seems to be both highly toxic when used individually and also able to augment the effects of other chemotherapeutics.

Treatment of many cell lines with Bortezomib induces the stabilization of the BH3-only proteins Bim and Bik, and MEFs deficient in Bik or Bim and Bik are less susceptibile to Bortezomib-induced cell killing.[34] Moreover, LNCaP prostate cancer cells treated with RNAi against Bim and Bik showed greatly reduced apoptosis in response to Bortezomib and TRAIL treatment. Stabilization of BH3-only proteins may, therefore, be a key mechanism in Bortezomib-induced cell death, and its ability to sensitize towards other stimuli (Figure 1).

On the flip side of the equation, inactivation of the NFκB survival pathway may also be an important component of the antineoplastic effects of this proteasome inhibitor. Bortezomib causes downregulation of several NFκB target genes including Bcl-2, A1, cIAP-2 and FLIP.[38] Multiple myeloma cells treated with Bortezomib have reduced binding activity to bone marrow stromal cells, suggesting that NFκB-mediated expression of adhesion molecules may be downregulated.[39] In addition to this correlative evidence, Bortezomib treatment increases IκB phosphorylation in pancreatic tumour cells, and decreases the binding of NFκB to DNA in response to treatment with death-inducing compounds including TNF and the chemotherapeutic CPT-11.[40] However, one study showed that these effects on NFκB gene targets were seen both in cells sensitive and highly resistant to Bortezomib treatment, suggesting that the latter drug kills tumour cells via multiple mechanisms.

Tumour vasculature also appears to be affected by Bortezomib treatment. Isolation of pancreatic tumours from Bortezomib-treated mice showed large areas of necrotic cell death both in tumours derived from cells sensitive to as well as cells resistant to Bortezomib-induced death.[40] Examination of the vasculature from these tumours showed a lower density of tumour microvessels and reduced secretion of VEGF. Decreased angiogenesis has also been observed in murine multiple myeloma chemotherapy models. Thus, Bortezomib may kill tumour cells either directly by causing apoptosis in the tumour, or indirectly by disrupting the blood supply to the tumour.

On the basic of these preclinical results, Bortezomib has entered into Phase I and II clinical trials and has shown very promising results for the treatment of refractory multiple myeloma. Clinical trials have now also shown effectiveness in treatment for non-Hodgkins and mantle cell lymphoma, and several solid tumour types.[35] This success in clinical trials has validated the concept of proteasome inhibitors as anticancer agents, and should pave the way for the development of further strategies for the inhibition of proteasome function for therapeutic gain.

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