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Review

The role of the ubiquitin-proteasome pathway in apoptosis

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Abstract

Coordinated intracellular protein degradation mediated by the ubiquitin-proteasome pathway is crucial to a vast array of cellular processes including orderly progression through the mitotic cycle. Similarly important to both the fates of individual cells, as well as to the normal function of multicellular organisms, is the process of apoptosis, or programmed cell death. Execution of this latter process has been known for some time to be intimately associated with the activity of caspases, a family of proteases related to interleukin-1-β-converting enzyme. Evidence is now accumulating, however, that the ubiquitin-proteasome system itself plays an important role in apoptosis, and some of the cellular pathways that are impacted upon by the proteasome, and may lead to apoptosis, are beginning to be dissected. This review provides a summary of the experimental basis by which components of the ubiquitin-proteasome pathway have been linked to apoptosis, and attempts are made to formulate a hypothesis about its role in this process.

Keywords: apoptosis; multicatalytic proteinase complex; programmed cell death; proteasome; ubiquitin

Abbreviations: Cdk, cyclin-dependent kinase; CLL, chronic lymphocytic leukemia; E1, ubiquitin activating enzyme; E2, (Ubcs) ubiquitin conjugating enzymes; E3, ubiquitin ligase; FMK, fluoromethylketone; Fas L, Fas ligand; Hsp, heat shock protein; IAP, inhibitors of apoptosis; ICE, interleukin-1 β-converting enzyme; ISM, intersegmental muscle; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor κB; NGF, nerve growth factor; PARP, poly (ADP-ribose) polymerase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand. Conventional single letter abbreviations are used to represent amino acids.

Introduction

The ubiquitin-proteasome pathway

The ubiquitin-proteasome system is the major extra-lysosomal pathway responsible for intracellular protein degradation in eukaryotes. It is composed of the ubiquitin-conjugating system and the 26S proteasome, the latter of which contains the multicatalytic proteinase complex, also known as the 20S proteasome, as its proteolytic core. Coordinated function of the ubiquitin conjugating system involves several classes of enzymes including a ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), ubiquitin ligase (E3), and also ubiquitin hydrolases. In combination with a target protein, the activity of this pathway results in mono- or polyubiquitination. Polyubiquitinated proteins are targets for degradation by the proteasome, a large multisubunit particle found in the nucleus and cytoplasm of eukaryotic cells.[1–7]

Function of the ubiquitin-proteasome pathway is necessary for cell growth by regulating the entry and exit to and from the mitotic cycle through timely degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors.[8,9] The proteasome is also associated with many other important functions, including degradation of transcription factors, short-lived regulatory proteins, antigen processing, and angiogenesis.[1–7,10] Studies have shown that the proteasome is absolutely necessary for cell survival and proliferation. Indeed, disruption of any one of 13 of the 14 genes which encode subunits that make up the inner 20S proteolytic core has been shown to be lethal to cells.[11]

Apoptosis

Apoptosis is an organized ATP-dependent form of cellular death which requires *de novo* protein synthesis and gene transcription, and is often therefore called programmed cell death. Morphologically it is characterized by cellular shrinkage and condensation of the nuclear contents, followed by plasma membrane ruffling and blebbing. The cell is then partitioned into membrane-bound apoptotic bodies containing various combinations of the original cellular contents, which are shed from the dying cells and phagocytosed by surrounding cells.

Programmed cell death is crucial in many normal aspects of the life of multicellular organisms. Disordered apoptosis is important in many disease processes including oncologic and rheumatologic phenomena, and thus accurate control of apoptosis is important not just to the normal development of an organism but also to its continued health and growth.[12–14] Three phases of apoptosis have been recognized, consisting of the initiation phase, the commitment phase, and the execution phase. This last phase generally involves the activation of soluble

proteins related to the interleukin-1 β-converting enzymes (ICE) which, because they cleave on the carboxyl side of aspartate residues and act as proteases, are often called caspases.[15] For reviews of the roles of these proteases in apoptosis, the reader is referred to[16–18]. Though the caspases have been the best known proteases which are involved in apoptosis, recent studies have revealed that the ubiquitin-proteasome pathway also has an important role in this process. Given the importance of the proteasome in intracellular protein turnover this was perhaps to have been expected, since one would anticipate that proteasome activity would be important in degrading the contents of cells undergoing apoptosis. Careful study of the proteasome, however, has revealed an unexpected and wonderful diversity of apparent roles. For example, in some cells proteasome inhibition promotes apoptosis, while in others it protects cells from apoptosis. This review will summarize the current body of experimental knowledge linking the ubiquitin-proteasome pathway to apoptosis, and attempt to synthesize a hypothesis about its role in this process.

Ubiquitination and apoptosis

Correlation of apoptosis with ubiquitination

One of the first reports linking the process of ubiquitination with apoptosis emerged from studies of intersegmental muscle (ISM) programmed cell death in the tobacco hawkmoth *Manduca sexta*. Hybridization of cDNA encoding a *Manduca* polyubiquitin gene to RNA isolated during muscle atrophy revealed a dramatic increase of polyubiquitin expression, and a parallel increase in protein-ubiquitin conjugates. Both of these findings were confirmed in tissue sections, and were noted to occur before morphological changes of programmed cell death.[19]

Further evidence for the involvement of ubiquitin in apoptosis came from studies of murine lymphocytes treated with teniposide, which induced a bright cytoplasmic ubiquitin staining pattern distinct from the nuclear immunofluorescence of control cells.[20] In the colonial ascidian *Botryllus schlosseri*, increased ubiquitin protein levels were noted in stomach, branchial basket, and intestinal tissues undergoing apoptosis.[21] Human lymphocytes exposed to γ-irradiation revealed increased ubiquitin immunofluorescence in cells with apoptotic nuclear morphologies 1–4 h after irradiation, even before visible chromatin changes. Polyubiquitin gene expression was increased 15 min after irradiation, and nuclear extracts contained increased amounts of newly synthesized ubiquitin.[22] In studies of thyroxine-induced apoptosis of cultured *Rana catesbeiana* tadpole tail tips, significant elevations of ubiquitin levels were demonstrated.[23] Dystrophin-deficient mice which developed apoptotic nuclear changes in muscle tissues increased expression of a polyubiquitin protein,[24] and also increased ubiquitin immunostaining and ubiquitin-protein conjugates in tibialis muscles, as did serum-deprived *cis*-platinum-treated myoblasts.[25] Muscle C3H/Sol8 satellite cells which were serum deprived showed dramatic increases in ubiquitin-protein conjugates after triggering apoptosis, though total protein levels appeared stable, and no activation of ubiquitin transcription was detected.[26] Such ubiquitin transcription was increased, however, in mouse RVC cells exposed to a proteasome inhibitor that induced apoptosis, while etoposide-induced apoptosis did not impact on ubiquitin transcription.[27] Increases in ubiquitin immunofluorescence and ubiquitin-protein conjugates have been noted in Ewing's sarcoma cells treated with radiation or lactacystin, a specific proteasome inhibitor.[28] Strong ubiquitin immunoreactivity was noted in the cerebellar external granule cell layer of Sprague-Dawley rats that were either irradiated or injected with methylazoxymethanol acetate, though, of the cells which were apoptotic, only about 15% showed this increased staining.[29]

Returning to *Manduca sexta* as a model system, increased cytoplasmic ubiquitin immunoreactivity was noted preceding the commitment to die of certain neurons in the abdominal ganglia.[30] In ISM tissue the increase in ubiquitin occurred in two phases: during the first phase, though the total size of the ubiquitin pool remained constant, there was a redistribution away from free ubiquitin towards conjugated forms; while during the second, slightly later phase, there was a tenfold increase in the total ubiquitin pool, and an increase in the fractional level of conjugation. Coincident with this increase there was a coordinated induction of E1, E2, and E3 conjugating activities, with the appearance of new, possibly cell death-specific E2 isoforms.[31]

More indirect evidence for the importance of the ubiquitin system in apoptosis came from studies of a novel murine gene termed Ft1 from a genomic area whose deletion resulted in various abnormalities in programmed cell death. This gene proved to have similarity to ubiquitin-conjugating enzymes.[32] Another protein with ubiquitin-conjugating activity, termed BRUCE, has been noted to have sequence similarities to the so-called IAP proteins, which act as inhibitors of apoptosis.[33] Finally, Nedd4, a widely expressed ubiquitin-protein ligase, is cleaved by caspases in several cell lines after induction of apoptosis.[34] It is likely that further study of these and other gene products will uncover additional links between apoptosis and the ubiquitination system.

While findings such as these argue for an important role of ubiquitination in apoptosis, other studies have not supported this conclusion. Dying cells in the *Drosophila* compound eye did not activate either ubiquitin gene transcription or protein expression.[35] Rat sympathetic neurons induced to undergo apoptosis by deprivation of nerve growth factor (NGF) did not activate the ubiquitin gene.[36,37] Differentiated PC12 pheochromocytoma cells deprived of NGF showed no increased ubiquitin mRNA after 48 h, though some subsequent increases seemed to occur at 72 and 105 h when, however, most cells were not viable.[36] In *B. schlosseri*, though increased ubiquitin immunostaining was seen, another antibody did not detect increased ubiquitin levels by immunoblotting.[21] In T-cells induced to undergo apoptosis by dexamethasone, ubiquitin expression was not noted to increase.[38] In *M. sexta*

abdominal ganglia, some neurons fated to die did not have increased ubiquitin immunostaining, while other neurons with high staining levels did not undergo apoptosis.[30] In *R. catesbeiana* tail tips treated with cycloheximide, ubiquitin levels rose only slightly, if at all, yet apoptosis still occurred, though to a lesser extent.[23] Rat T24 glioma cells and 476-16 rat trigeminal neurinoma cells induced to undergo apoptosis by transforming growth factor β_1 had comparable levels of free ubiquitin and ubiquitin-conjugated proteins to control cells. Levels of ubiquitinated histone 2A were decreased, arguing that a specific alteration of the ubiquitin system rather than a general derangement was seen in the condensation of chromatin accompanying apoptosis, as it was in metaphase chromosomes.[39] Decreased levels of ubiquitinated histone 2A in mouse lymphoma RVC cells undergoing apoptosis were also detected after proteasome inhibition.[27] Furthermore, some of the model systems which were used to show correlations between ubiquitin activation and apoptosis did not display all of the features now classically associated with this process. Programmed cell death in *M. sexta* is not accompanied by the generation of a ladder of chromatin DNA fragments, and might therefore involve a slightly different death program.[38] Apoptotic cells and debris in *B. schlosseri* are recognized and ingested by blood macrophages or intraepithelial phagocytes,[21,40] rather than by their normal surrounding neighbors.

Interference with ubiquitination and apoptosis

Several studies have interfered with ubiquitination and then evaluated the impact on apoptosis. In human lymphocytes, ubiquitin antisense oligonucleotides inhibited both the increase in protein ubiquitination and the triggering of apoptosis by γ-irradiation.[22] A murine fibroblast cell line with a temperature-sensitive E1 enzyme that cannot activate ubiquitin at the non-permissive temperature has also been studied. These cells underwent apoptotic changes at this temperature, which could be antagonized by Bcl-2 expression, and apoptosis appeared to be independent of caspase activation. Overexpression of Bcl-2 also relieved the cell cycle arrest normally present in this cell line at the non-permissive temperature.[41] This finding is surprising given the importance of the ubiquitin-proteasome pathway in cell cycling, and may suggest a close relationship between Bcl-2 and this pathway.

The overall weight of the evidence does seem to imply that ubiquitination of proteins has an important role in the apoptotic process. This especially seems to be the case in the programmed cell death of tissues where extensive degradation of intracellular proteins is necessary, such as in the ISMs of *Manduca sexta*. Most of the models that have been examined in which this did not seem to be the case occurred in systems derived from thymocytes and neural tissues which, as is noted subsequently, commonly differ in their requirement for ubiquitin-proteasome activation for apoptosis. Studies which have shown that interfering with ubiquitination results in apoptosis suggest a causal role, but it is still possible that activation of the ubiquitin conjugating system is a secondary phenomenon of apoptosis.

The proteasome and apoptosis

Correlation of apoptosis with proteasome function

The involvement of ubiquitin in apoptosis certainly implied that the proteasome, the degradatory machinery for proteins targeted by ubiquitination, was an important part of the process as well. Such a correlation was first demonstrated using the model system of *M. sexta* ISM programmed cell death. Studies following the abundance of proteasome subunits revealed a fivefold increase in levels with the onset of programmed cell death, and several ATPase regulatory subunits increased as well. Increased function of several of the proteolytic activities of the proteasome was noted, especially in the 26S proteasome fractions.[42] Some changes were also noted in non-ATPase subunits, and this proteasome remodeling did not seem to occur in muscles not undergoing apoptosis.[43] Subsequent work revealed that an ATPase regulatory subunit dramatically rose in abundance prior to programmed cell death, though smaller increases were noted in muscle tissue not destined for apoptosis.[44] Another group studying this model system reported that the abundance of the multicatalytic proteinase complex rose approximately eightfold as cells became committed to die. There was also a relative increase in at least two of the proteasome activities, which correlated with the appearance of up to four novel proteasome subunits.[45]

Other investigators have examined the distribution of proteasomes in cells undergoing apoptosis. In an immortalized granulosa cell line proteasomes migrated from the nucleus to apoptotic blebs during programmed cell death induced by activation of p53.[46]

Not all studies, however, have revealed changes in the proteasome with the onset of programmed cell death. Proteasomes were present in both the cytoplasmic and nuclear remnants of apoptotic MR65 human squamous lung carcinoma cell lines. Though the intensity of staining was greater than in control cells, the apoptotic cellular volumes were smaller, and the authors concluded that the concentration of proteasomes was probably similar.[47] In *M. sexta* dorsal midline motoneurons, immunostaining was not consistent with an increase in the multicatalytic proteinase complex, and no overlap was noted between increased levels of ubiquitin-protein conjugates and high levels of proteasome reactivity in dying neurons, while other cells with low ubiquitin staining had intense proteasome reactivity.[48] From a functional perspective, studies with primary thymocytes induced to undergo apoptosis revealed that the overall rate of proteolysis was comparable to control cells, though proteasome-specific activity was not assayed.[49] Similar findings were noted by a second group, who also found that 20S proteasome activity decreased during this process.[50] In contrast, others noted that proteasomal activity increased in thymocytes treated with dexamethasone or etoposide.[51] P19 murine embryonal carcinoma cells undergoing apoptosis after treatment with retinoic acid were found to have unchanged absolute proteasome levels, but ATP-dependent proteasome activity increased twofold.[52] A concentration dependence was noted in studies of low doses of a proteasome inhibitor, which were sufficient to induce apoptosis and had little

overall effect on protein degradation, but turnover of some very short-lived proteins such as I-κB was impacted upon. At higher concentrations, however, significant effects were noted even with proteins that had longer half-lives.[53]

Interference with proteasome function and apoptosis

Stimulation of apoptosis With the discovery of inhibitors of the proteasome, it became possible to more directly evaluate its role in apoptosis. The first such study used lactacystin, a *Streptomyces* product which binds and inhibits several proteasome activities (reviewed in[54]). Human monoblast U937 cells grown in the presence of this agent had decreased viability, underwent morphologic changes suggestive of apoptosis, and their chromosomal DNA revealed fragmentation and laddering.[55] Subsequent investigators confirmed the induction of apoptosis in U937 cells using peptidyl aldehyde proteasome inhibitors.[53]

Studies evaluating the impact of a cell-permeable peptidyl aldehyde proteasome inhibitor on human T-cell leukemia MOLT-4 cells and mouse lymphocytic leukemia L5178Y cells showed that apoptosis was induced.[56] Peptidyl aldehyde proteasome inhibitors and/or lactacystin have been shown to induce apoptosis in a variety of cell lines, including mouse RVC lymphoma cells;[27] proliferating Rat-1 fibroblasts and proliferating or nonproliferating PC12 cells;[57] unsynchronized HL60 promyelocytic leukemia cells;[58] DO.11.10 T-cell hybridoma cultures;[59] nontransformed mouse hepatocytes;[60] cells from patients with chronic lymphocytic leukemia;[61] serum-starved Chinese hamster ovary cells;[62] J774.A1 murine macrophage-like cells treated with lipopolysaccharide or infected with various Yersinia bacterial strains, and HeLa cells also treated with TNF-α;[63] Jurkat cells and activated T-cells;[64] 293 human kidney cells;[53] primary and transformed rodent fibroblasts, human lymphoblasts, and human-derived Burkitt's lymphoma cells.[65] Some of these apoptosis-inducing inhibitors have a broad specificity, and seem to inhibit several of the proteolytic activities of the proteasome. Investigators who have used peptidyl aldehydes which, at least *in vitro*, have a high degree of specificity towards the chymotrypsin-like activity of the proteasome, noted that these agents also induced apoptosis.[57,65] Thus, it may be possible to inhibit only a portion of the proteasome and induce programmed cell death.

Inhibition of apoptosis Though many studies used proteasome inhibitors to demonstrate the induction of apoptosis, others found that these agents inhibited programmed cell death. Using primary thymocytes from BALB/c mice, several peptidyl aldehyde proteasome inhibitors as well as lactacystin antagonized the induction of cellular apoptosis due to dexamethasone, γ-radiation, or phorbol esters. The addition of staurosporine or phorbol ester with the calcium ionophore A23187, however, resulted in apoptosis which could not be antagonized.[49] Similar inhibition of thymocyte apoptosis by proteasome inhibitors was noted for dexamethasone-induced cell death;[50] for etoposide-induced apoptosis, though not for calcium ionophore- or staurosporine-induced cell death;[66] and for both dexamethasone- and etoposide-induced apoptosis.[51] In experiments using rat sympathetic neurons induced to undergo apoptosis by NGF withdrawal, inhibitors of both cysteine proteinases and the proteasome promoted neuron survival.[67] Subsequent work showed that lactacystin could not inhibit apoptosis induced in neurons by microinjection of constructs expressing Bax or Bak.[68] Using a cell-free system where apoptosis was induced in HL60 nuclei by the addition of cytosol from camptothecin-treated, apoptotic HL60 cells, a peptidyl aldehyde inhibitor was noted to inhibit DNA fragmentation, though only at extremely high concentrations of 500 μM, and a similar activity was not demonstrated with whole cells. The latter were, in fact, induced to undergo apoptosis by this proteasome inhibitor.[69] While studies of these various inhibitors and their impact on apoptosis have provided invaluable insights into this function of the proteasome, these reagents do have their limitations. Though some peptidyl aldehydes have specificity for various activities of the proteasome,[70,71] other inhibitors have activity against cellular thiol proteases, such as the calpains.[72–74] This potential inhibitory activity needs to be considered, especially since a recent study implies that the calpains themselves may have a role in apoptosis.[75] Even lactacystin, one of the most selective proteasome inhibitors available, inhibits cathepsin A under certain conditions.[76] Nonetheless, the evidence is fairly overwhelming in supporting an important role for proteasome-mediated proteolysis in the apoptotic process.

Mechanisms of apoptosis and the proteasome

Stimulation *vs* inhibition of apoptosis

One of the more curious aspects of the role of the proteasome in programmed cell death is the contradictory impact of proteasome inhibition, which results in promotion of apoptosis in most cell lines, but in a few others it protects cells from apoptosis. Since protection has been found predominantly in primary cell cultures such as thymocytes[49–51,66] and neurons,[67,68] these and other investigators have postulated that the differentiated, non-dividing status of these cells is an important determinant, and that apoptosis was only induced in actively proliferating cell lines. This is supported by results showing that proliferating Rat1 cells were more sensitive to apoptotic induction than quiescent Rat1 cells;[57] that noncycling, differentiated HL60 cells were less sensitive than unsynchronized undifferentiated HL60 cells;[58] and that resting T-cells were less sensitive than activated T-cells.[64]

Another factor that may be involved is the activation of c-Jun N-terminal kinase (JNK) and the heat shock protein Hsp72. With short exposures to low concentrations of a peptidyl aldehyde inhibitor JNK was activated only temporarily, while Hsp72 activation was longer lasting. Such cells were then resistant to apoptotic stimuli that required JNK activation, such as heat shock and ethanol treatment, though apoptosis could still be induced and even

potentiated with agents which used alternative pathways, such as TNF. With longer exposures of these cells to the inhibitor, JNK activation was more prolonged and led to apoptosis.[53] Finally, in studies of Sindbis Virus-induced NF-κB induction and apoptosis, a concentration dependence was noted for several proteasome inhibitors. At very low concentrations these agents were able to inhibit both of these processes without cytotoxicity, while at higher concentrations the compounds alone induced apoptosis.[77] Evaluation of such concentration dependence and JNK pathway activity in some of the cellular models noted above could provide valuable experimental verification of these possibilities.

The proteasome and the apoptotic cascade

Once the proteasome's involvement in apoptosis was established, investigators pursued experiments trying to determine the position that this protease occupied in the apoptotic process. The impact of combinations of TNF and peptidyl aldehyde proteasome inhibitors as well as lactacystin was studied on cleavage of poly (ADP-ribose) polymerase (PARP) by the CPP32 protease, which occurs during apoptosis, in U937 monoblasts. These inhibitors enhanced PARP cleavage and CPP32-like activity caused by TNF treatment, while themselves inducing CPP32-like activity in the absence of TNF only after prolonged exposure times. ICE-like activity was not stimulated, but a peptide inhibitor of CPP32-like activity both inhibited the generation of CPP32-like activity, and the decreased cell viability induced by combinations of TNF and a proteasome inhibitor.[78] Subsequent investigations confirmed PARP cleavage in U937 cells after proteasomal inhibition.[53] In mouse lymphoma RVC cells, however, while an inhibitor of CPP32-like activity did decrease DNA fragmentation induced by proteasome inhibition, ICE inhibitors did not impact on this process.[27] HL60 cells exposed to proteasome inhibitors showed decreased levels of CPP32 precursor and increased PARP cleavage and, as in the RVC cell system, apoptosis was sensitive to a CPP32 inhibitory peptide but not an ICE inhibitory peptide.[58] PARP cleavage has also been demonstrated in Ewing's sarcoma cells treated with lactacystin.[28]

In primary thymocytes where proteasome inhibitors antagonized apoptosis, PARP cleavage significantly decreased, though the inhibitors themselves stimulated low levels of cleavage in some situations.[49] PARP cleavage was decreased by proteasome inhibitors in rat sympathetic neurons, while in murine macrophages stimulated by lipopolysaccharide these inhibitors blocked IL-1β and ICE processing.[67] Caspase 3-like activity stimulated by etoposide-treated thymocytes was decreased by proteasome inhibition.[66]

Recently published work investigated the impact of proteasome inhibition on mitochondrial membrane potentials in thymocytes induced to undergo apoptosis by dexamethasone or etoposide. Several proteasome inhibitors prevented dissipation of the mitochondrial transmembrane potential, post-mitochondrial generation of superoxide anions, loss of nuclear DNA, and exposure of phosphatidyl-serine. Additionally, neither bongkrekic acid nor a caspase inhibitor prevented activation of the proteasome by dexamethasone or etoposide, though they both prevented more downstream processes. Taken together the data was interpreted as demonstrating that proteasomes acted upstream at a pre-mitochondrial and pre-caspase stage of apoptosis.[51]

To establish the impact of various antiapoptotic proteins, Rat-1 cells overexpressing either Bcl-2 or CrmA were studied, and found to be resistant to proteasome inhibitor-mediated apoptosis.[57] Bcl-2 also inhibited proteasome inhibitor-mediated apoptosis in Ewing's sarcoma cells.[28]

The available evidence supports the possibility that proteasomes act upstream of both the caspase cascade and of mitochondria in the apoptotic process, whether that activity results in the promotion or inhibition of apoptosis. This form of apoptosis does seem to require at least some portion of the caspase pathway, though ICE-like activity seems not to be involved, and caspase-independent activation of apoptosis by inhibition of the ubiquitin-proteasome pathway has been reported as well.[41] Studies of apoptosing P19 murine embryonal carcinoma cells noted that, in addition to increased proteasomal activity, ICE-like activity was increased more than sixfold. When purified this activity was sensitive to both proteasome inhibitors and ICE inhibitors, but was decreased only with anti-proteasome IgG, and associated with a 700 kDa multisubunit particle. Such findings suggest that the proteasome might itself have an ICE-like activity, though since the calculated K_m and K_i values were several orders of magnitude higher than for purified ICE, the physiological relevance of such activity is unclear.[52] Others have noted that the caspase inhibitor VAD-FMK impacted upon 26S proteasome activity, but 20S activity was stimulated, and both changes required high inhibitor concentrations of 100 μM, again leading to questions about its physiological relevance.[50] Thus, the proteasome itself may under certain conditions have a caspase-like activity which might stimulate apoptosis, or acts on proteins which themselves trigger or inhibit this cascade. The last of these three possibilities has been invoked in studies of c-Fos degradation, which occurs through the proteasome, and seems to be an early, Bcl-2 regulated step in the induction of apoptosis in some cells.[79]

The proteasome and the cell cycle

Coordinated proteolytic activity of the ubiquitin-proteasome system has proven to be crucial to normal progress through the cell cycle. Early studies evaluating the consequences of inhibition of this function revealed mutations in a ubiquitin-activating enzyme in Chinese hamster ovary cells led to an arrest at G_2/M,[80] as did yeast mutations which prevented the assembling of ubiquitin chains.[81] Inhibition of the proteasome resulted in cell-cycle arrest at the G_1/S boundary in Chinese hamster ovary cells[82] as well as in Neuro 2A and MG-63 osteosarcoma cells,[83] while HeLa cells arrested at G_2/M,[84] and other studies noted cellular arrest at both G_1/S and G_2/M.[85] This is by no means a complete review of the effects of proteasome inhibition on progression through the cell cycle, however, which is beyond the scope of this work. The reader is referred to several recent reviews.[8,9]

In attempts to correlate the cell cycle position of cells exposed to proteasome inhibitors with the onset of apoptosis, mouse lymphoma RVC cells were found to be enlarged during apoptosis, rather than shrunken. Cell cycle analysis showed that most of the cells accumulated at either a sub G_1/G_0 DNA content consistent with apoptosis, or in the G_2/M phase.[27] Unsynchronized HL60 cells which underwent apoptosis after proteasome inhibition were noted to be predominantly in the G_1 phase.[58] Proteasome inhibition could result in arrest at G_1/S by virtue of accumulation of either p53 or p27, or both, the incoordinated degradation of cyclins D and E, or the requirement of proteasome activity for cdk 2 activity.[64] Arrest at G_2/M could result from inhibition of degradation of cyclin B (Figure 1). One mechanism commonly invoked to explain the triggering of apoptosis involves the generation of conflicting signals for both cellular growth and cell cycle arrest, which can only be resolved by cellular self-destruction.

The p53 pathway

The important role of p53 in tumorigenesis and programmed cell death (reviewed in[86–88]), as well as the fact that p53 is degraded by the ubiquitin-proteasome pathway[89] (reviewed in [90]), made it one of the first proteins examined for possible involvement in proteasome inhibitor-mediated apoptosis. MOLT-4 cells treated with a proteasome inhibitor were noted to undergo apoptosis and, in parallel, to accumulate p53,[56] while a cell line containing a temperature-sensitive E1 enzyme also accumulated p53 in parallel with apoptosis.[41] p53 levels increased in Rat-1 and PC12 cells exposed to proteasome inhibitors, and this p53 was believed to be transcriptionally active because expression of both p21 and Mdm-2, which are targets of p53,[86–88] increased as well. Furthermore, when these two cell lines were transfected with a vector expressing a dominant-negative p53 mutation, they became relatively resistant to this apoptotic induction.[57]

Involvement of the p53 pathway in this apoptosis seems, therefore, very likely, at least in some cells. Increased levels of p53 would transactivate expression of the cyclin-dependent kinase inhibitor p21, whose action can mediate arrest of cycling cells in G_1 by preventing phosphorylation of pRb and activation of E2F, and itself is degraded by this pathway.[91] Apoptosis of such arrested cells could well be mediated by a combination of upregulation of the Bax gene, a pro-apoptotic target of p53, and downregulation of the anti-apoptotic protein Bcl-2, which is repressed by p53.[86] Moreover, there are recent reports that Bax itself is degraded through the ubiquitin-proteasome pathway.[91] Thus, proteasome inbibition could induce a cascade of pro-apoptotic signals by virtue of its activity on the p53 pathway (Figure 1). Mdm2 is also degraded by the proteasome[91] and its accumulation might therefore be expected to antagonize this process. Since it would do so by stimulating p53 degradation through the proteasome,[86–88] however, whose activity would be blocked, this negative feedback loop would be inactive. The role of another protein which seems to be involved in this pathway, the $p19^{ARF}$ tumor suppressor,[92,93] has not yet been investigated. Increased p53 levels alone, however, must not be sufficient to induce apoptosis, since such accumulation is seen after proteasome inhibition of thymocytes, yet these cells are prevented from undergoing cell death induced by etoposide.[66] Also, proteasome inhibition is sufficient to induce apoptosis in HL60 cells, which are p53-null,[58] and U937 cells undergo apoptosis without detectable accumulation of p53.[53]

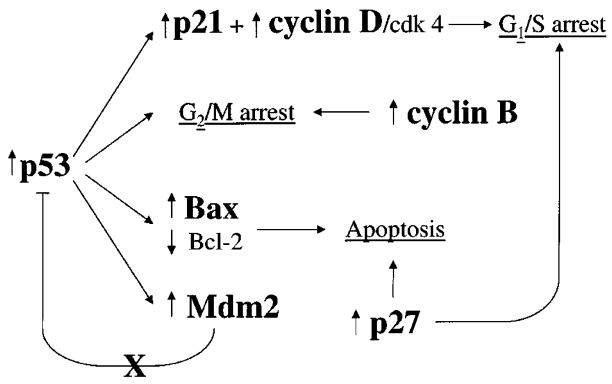

Figure 1 The p53 pathway, proteasome inhibition, and apoptosis. Proteasome inhibition results in the accumulation of p53, which is normally degraded in a proteasome-dependent manner. This could result in p21 transactivation which, in combination with cyclin D and cdk 4, will result in G_1/S arrest. Increased p53 activity also results in up-regulation of Bax and down-regulation of Bcl-2, thereby favoring the accumulation of Bax/Bcl-2 heterodimers, which induce apoptosis, rather than Bcl-2/Bcl-2 homodimers, which antagonize apoptosis. Mdm2 is transactivated and would normally increase degradation of p53, but since this also occurs in a proteasome-dependent manner this negative regulatory pathway is blocked. p53 accumulation may also result in G_2/M arrest, and the combination of apoptosis, G_1/S arrest, and G_2/M arrest is in fact seen as a result of proteasome inhibition (see text). In addition to the effects on p53, since the proteasome is involved in the degradation of many of the downstream proteins (indicated in bold lettering), there is a potentiation effect with, for example, increased p21 levels being due to both increased p53 levels and decreased p21 degradation. The effects on downstream proteins also mean that this pathway may be important even in cells with mutated, transcriptionally-inactive p53. Contributions to the processes of apoptosis and cell cycle arrests from p27 and cyclin accumulation are indicated as well

p27 and other cell cycle regulatory proteins

Due to the impact of proteasome inhibition on cell cycling through the G_1/S and G_2/M boundaries, other proteins which impact upon these transition points are being investigated. The critical role that $p27^{Kip1}$ plays in cell cycle regulation makes it an attractive candidate as an important pathway in proteasome inhibitor-mediated apoptosis. Given its function as an inhibitor of cdk2 and cdk4, accumulation has been noted to result in cell cycle arrest at the G_1/S phase,[90] while overexpression can result in apoptosis.[94,95] Since $p27^{Kip1}$ is known to be degraded in a ubiquitin- and proteasome-dependent manner,[96] its accumulation after proteasome inhibition could result in similar cellular effects (Figure 1). Several investigators have noted accumulation of $p27^{Kip1}$ in parallel with apoptotic changes, most notably in HL60 cells[58] and HeLa cells[91] exposed to proteasome inhibitors. Similarly, $p27^{Kip1}$ accumulates in fibroblasts at a non-permissive temperature undergoing apoptosis due to the presence of a

temperature-sensitive E1 enzyme.[41] It is not yet clear, however, whether accumulation of $p27^{Kip1}$ is an epiphenomenon of inhibition of the ubiquitin-proteasome pathway, or if it might be causative in the apoptotic process.

The orderly synthesis and proteasome-mediated degradation of cyclins is important in cellular progression through the cell cycle,[8,9] and cell cycle arrest followed by apoptosis due to proteasome inhibition could well involve effects on cyclin levels (Figure 1). Several investigators have noted increases in levels of cyclins D, E, and B in association with apoptosis, most recently in HeLa cells.[91] Once again, however, experimental verification of a causative role for these phenomena in the apoptotic process is lacking at this time.

The NF-κB cascade

NF-κB is one member of a large family of transcription factors found in the cytoplasm, but present in an inactive form bound to one of several inhibitory IκB proteins. Upon activation of this cascade IκB is phosphorylated, ubiquitinated, and degraded by the proteasome, releasing NF-κB, which translocates to the nucleus to act as a transactivator. This function seems to stimulate some pathways that promote cell death, while other pathways promote cellular survival, possibly through activation of IAP-like proteins.[97,98] Interference with this system by proteasome inhibition would therefore seem to have the possibility of impacting upon apoptosis, especially since proteasome function is also involved in activation of the IκB kinase and processing of the precursor of NF-κB (Figure 2) (recently reviewed in [99–101]).

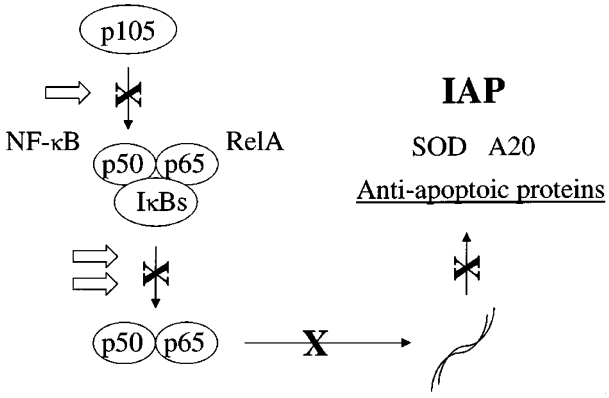

Figure 2 The NF-κB pathway, proteasome inhibition, and apoptosis. A precursor form of NF-κB, p105, is processed in a ubiquitin-proteasome-dependent manner to the mature p50 form. This form is present in the cytoplasm as a dimer with, for example, p65 RelA, and bound to inhibitory IκB proteins. With activation of this pathway, IκB protein are phosphorylated by an IκB kinase which is activated in a proteasome-dependent step, and then degraded again in a proteasome-dependent manner to free the active heterodimeric complex. After binding to DNA one of the functions of these transcription factors is to induce an anti-apoptotic activity, which is likely due to inhibitors of apoptosis (IAP) proteins and also possibly superoxide dismutase (SOD) and A20. Since there are several proteasome-dependent steps in the activation of NF-κB (marked with bold arrows), inhibition of the multicatalytic proteinase complex (indicated by bold X's) effectively shuts this pathway down, potentially decreasing the levels of several proteins with anti-apoptotic activities

Confirmatory evidence of the involvement of this pathway was presented in a study of T-cell death induced by binding with anti-CD3, which requires activation of the Fas ligand (Fas L) and upregulation of the Fas gene (recently reviewed in[102]). Peptidyl aldehydes and lactacystin inhibited this activation-induced cell death by virtue of their inhibition of IκBβ degradation and NF-κB translocation, and inhibition of both FasL and Fas mRNA as well as Fas surface expression.[59] Studies such as these imply that blockage of NF-κB activation by proteasome inhibition should protect cells from apoptosis. A constitutive level of NF-κB activity has been found to be important for B cell survival, however, since inhibition of this function induces apoptosis.[99,103,104] Supporting the role of proteasome inhibition in this process, inhibition of NF-κB translocation in nontransformed mouse hepatocyte cells and AML hepatocytes by lactacystin was demonstrated to induce apoptosis.[60] CLL cells with high levels of NF-κB activity compared to normal lymphocytes were noted to undergo increased apoptosis after inhibition of this activity with lactacystin.[61] Decreased levels of nuclear p65 immunoreactivity and NF-κB binding were noted to correspond to the induction of programmed cell death by injection of a proteasome inhibitor into rats.[105] Other investigators who have experimentally linked these pathways noted that blocking NF-κB activation with a proteasome inhibitor enhanced apoptosis induced by TRAIL.[106]

In addition to these more direct proofs of the role of NF-κB in proteasome inhibitor-mediated apoptosis, other findings suggest a close link between these two systems. Yeast two hybrid screens searching for proteins which interact with intracellular portions of the p55 TNF receptor identified a novel protein with homology to a yeast subunit of the 26S proteasome.[107] Similar screens using the intracellular domain of Fas/APO-1 (CD95) revealed the binding of a protein which inhibited apoptosis induced by anti-Fas antibody, termed sentrin, that had some sequence homology to ubiquitin.[108] Another set of investigators using this Fas/APO-1 domain isolated a clone with striking homology to E2 ubiquitin-conjugating enzymes. This protein, dubbed UBC-FAP, was identical to the cloned human UBC HsUbc9,[109] and the mouse homologue was later identified from a mouse embryo cDNA library.[110]

The available evidence is certainly suggestive of an important role for interference with normal activity of the NF-κB family of transcription factors and proteasome inhibitor-mediated apoptosis. The inability of proteasome inhibitors to antagonize apoptosis in thymocytes treated with staurosporine,[49] which does not require involvement of NF-κB for apoptosis,[111] is further indirect evidence supporting this possibility. A similar mechanism for apoptotic induction involving prevention of translocation of NF-κB to the nucleus has been postulated for apoptosis induced in murine macrophages by infection with strains of Yersinia.[63]

Additional candidates

The broad range of proteins whose levels are impacted upon by the ubiquitin-proteasome pathway makes any of them

which might be involved in apoptosis potential candidates for investigation of the mechanism behind this phenomenon. Topoisomerase IIα, for example, is subject to degradation by this pathway during the course of adenovirus-mediated apoptosis.[112,113] Since degregulated expression of topoisomerase IIα is now being reported to result in apoptosis,[114] its accumulation as a result of proteasome inhibition may contribute to this process. It is very likely that, as the understanding of apoptosis grows, additional pathways will be recognized as being impacted upon by proteasome inhibition and contributing to programmed cell death.

Implications

Cancer therapy

The ability to induce apoptosis in tumor cell lines is an attractive feature for any potential anti-neoplastic agent. One might think, however, that since the proteasome is present in all cells, and that its coordinated activity is so important, inhibition would result in apoptosis of all cells. Surprisingly, there are reports of preferential apoptosis being induced in neoplastic cells. In a comparison of normal human lymphocytes with CLL cells, lactacystin induced much more apoptosis in the neoplastic cells.[61] Apoptosis generated in c-*myc*-transformed, EBV-immortalized human lymphoblasts with short exposures to a proteasome inhibitor was noted to occur up to 40-fold or more efficiently than in controls.[65] There are also indications that apoptosis generated by proteasome inhibition is complementary to other methods of inducing apoptosis. Studies evaluating human T-cell leukemia MOLT-4 cells and mouse lymphocytic leukemia L5178Y cells showed that combinations of a proteasome inhibitor with irradiation resulted in additive levels of apoptosis.[56] Jurkat cells exposed to combinations of proteasome inhibitors and an anti-Fas antibody had higher levels of apoptosis than either treatment alone.[115] In cells from patients with CLL which resisted apoptosis despite treatment with TNF, cellular sensitivity was restored after treatment with lactacystin.[61] Inhibition of NF-κB activation with a proteasome inhibitor enhanced apoptosis caused by TRAIL, anti-APO-1, TNFα, and doxorubicin, and cell lines as well as primary leukemia cells resistant to doxorubicin-induced apoptosis could be sensitized with this proteasome inhibitor.[106] Finally, proteasome inhibition may be beneficial in tumor therapy not just by the induction of apoptosis, but by other mechanisms as well. In support of this possibility is the recent finding that inhibition of the proteasome impacted upon angiogenesis.[10]

Further evaluation of the utility of proteasome inhibition for treatment of malignant conditions will require *in vivo* testing. One such report describing the use of peptidyl aldehyde inhibitors in a murine model of human Burkitt's lymphoma has recently appeared. Statistically significant tumor regression, tumor growth delay, and *in vivo* apoptosis were demonstrated after a single administration of a proteasome inhibitor without apparent toxicities.[65] Such results are encouraging for the possible use of a proteasome inhibitor in anti-neoplastic therapy, but extensive testing still awaits any potential candidate drugs.

Conclusions

Through the work of many investigators it is now clear that the ubiquitin-proteasome system has an important role to play in the cellular process of apoptosis. In most cell lines interference with this pathway triggers apoptosis, though in primary cells such as thymocytes and neural cells such interference can protect from apoptosis. This difference may be due to the proliferative state of these tumor-derived cell lines, the concentrations of inhibitory agents which are used, or to other factors such as differential activation of kinases such as JNK and certain heat shock proteins. The proteasome seems to impact upon the apoptotic process at a point upstream of the caspase cascade, and also upstream of mitochondria, although some reports are consistent with the involvement of a caspase-independent mechanism. Many cellular proteins that have important functions in apoptosis are degraded through the ubiquitin-proteasome system, and several of these have been examined as possibly contributing to proteasome inhibitor-mediated apoptosis. There is solid evidence that the p53 system as well as the NF-κB family of transcription factors are impacted upon by such inhibitors, and that these changes contribute to the triggering of apoptosis. Other proteins such as the cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors are perturbed, and the resulting effects on the cell cycle are probably involved with apoptosis as well. Though some of these and other pathways may play major roles in this programmed cell death, it is likely that this apoptosis as a result of interference with ubiquitin- and proteasome-dependent proteolysis occurs due to contributions from many pathways. From a teleological standpoint, pro-apoptotic proteins should have short half-lives since cells would want to carefully control their levels. In a normal cell the levels of pro- and anti-apoptotic proteins and their activities are likely to be balanced. When the half-lives of short-lived pro-apoptotic proteins such as p53, Bax, and p27 are prolonged by even transient proteasome inhibition, however, their absolute levels and activities increase. In contrast, the levels and activities of some anti-apoptotic proteins may not be significantly changed because these proteins, such as Bcl-2, normally have longer half-lives. Other proteins with both pro- and anti-apoptotic functions, such as NF-κB, seem to have decreased anti-apoptotic activity, perhaps due to decreased levels of IAP. The resulting imbalance favoring apoptosis may be enough to activate programmed cell death. This ability to promote apoptosis, especially in transformed cells may indicate that proteasome inhibition alone, or in combination with more traditional chemotherapy, has a role to play in antineoplastic therapy.

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