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Review

Proteasomes in apoptosis: villains or guardians?

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Abstract. The proteasome (multicatalytic proteinase complex, prosome) is a major cytoplasmic proteolytic enzyme, responsible for degradation of the vast majority of intracellular proteins. Proteins degraded by the proteasome are usually tagged with multiple ubiquitin moieties, conjugated to the substrates by a complicated cascade of enzymes. Over the last years, evidence has accumulated that changes in the expression and activity of the different components of the ubiquitin-proteasome system occur during apoptosis. Proteasome inhibitors have been used to induce apoptosis in various cell types, whereas in others, these compounds were able to prevent apoptosis induced by different stimuli. The proteasome mediated step(s) in apoptosis is located upstream of mitochondrial changes and caspase activation, and can involve in different systems Bcl-2, Jun N-terminal kinase, heat shock proteins, Myc, p53, polyamines and other factors.

Key words. Proteasome; ubiquitin; apoptosis; programmed cell death; caspases.

Introduction

Two complicated proteolytic systems are involved in the control of cell death: the caspase family of cytsein proteases and the ubiquitin-proteasome degradation system [1]. Whereas the role of caspases in apoptosis is well established, and consists of control of the execution phase [2], little is known about the exact role of the ubiquitin-proteasome system in programmed cell death [3–6].

The movement of a cell through the sequential phases of apoptosis is accompanied by a progressive decrease in cell size with loss in protein mass. Since proteasomes are responsible for the degradation of most cytoplasmic and nuclear protiens [7], it is logical to assume that they are responsible in great part for this protein breakdown. Indeed, a recent report confirms this point of view, showing activation of the proteasome system during apoptosis [8]. Nevertheless, others point out that during apoptosis the activity of proteasomes can be downregulated [9].

Multiple studies show that proteasomes take part in the apoptotic process, standing often at opposite sides of the barricade, sometimes acting as cell guards and protectors, and sometimes as villains and murderers. It is tempting to speculate that there exists some balance between the pro- and antiapoptotic activities of the proteasomes in the cells. This balance is preset in different cell types either in favor or against the apoptotic process, but even in a single-cell type it can be switched by processes like differentiation [10], availability of growth factors [11] and the degree of proteasome inhibition [12]. It also varies depending on the type of proapoptotic stimuli involved.

Ubiquitin in apoptosis

Increased ubiquitin immunoreactivity in different inclusion bodies found in neurodegenerative disorders suggested some role for ubiquitin in neuronal cell death; however, it was not clear whether it occurs through

necrotic or apoptotic changes. First evidence of the involvement of the ubiquitin system in apoptosis came from the study of programmed cell death in intersegmental muscles in the larval hawkmoth *Manduca sexta*. Schwartz and colleagues have shown that there is a dramatic increase in polyubiquitin gene expression [13]. Several other early studies followed.

In γ-irradiated lymphocytes, apoptosis involved an increase of ubiquitin messenger RNA (mRNA) and ubiquitination of nuclear proteins. Ubiquitin antisense inhibition considerably diminished the proportion of apoptotic lymphocytes after γ-irradiation, which clearly implied an active role for this system in cell death [14]. Increased ubiquitin immunoreactivity has been correlated with cell death in the stomach of the ascidian *Botryllus schlosseri* [15], neuronal apoptosis in amyotrophic lateral sclerosis [16] and cell death in the thyroxin-treated tadpole tail [17]. It was also observed in apoptotic neurons in *M. sexta*; however, some specific groups of surviving neurons involved in neuroendocrine function also displayed high ubiquitin immunoreactivity [18]. An increase in expression of polyubiquitin genes occurred early during apoptosis of RVC lymphoma cells caused by a set of proteasome inhibitors [19]. Radiation-induced apoptosis in Ewing's sarcoma cells [20] and MCF-7 breast carcinoma cells led to accumulation of high molecular mass ubiquitin-protein conjugates. In the latter case, two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) and Western analysis of proteins identified a set of isomeric ubiquitinated conjugates located at a pI range of 4.2–4.6 and an M_r of ~ 30 kDa [21].

Strikingly, another set of experiments has shown little or no relationship between apoptosis and ubiquitin expression. There have been no changes in ubiquitin mRNA during neuronal apoptosis induced by Nerve growth factor (NGF) deprivation [22]. The cellular level of free ubiquitin and the overall pattern of ubiquitin-conjugated proteins were unaltered in apoptotic glioma and Schwannoma cells [23]. Ubiquitin expression was also not changed during luteal apoptosis in marmoset ovaries [24] or in apoptotic ommatidial cells in the fruit fly [18].

Insect muscle cell death

Since the first report [13], intersegmental muscle cell death in the larval hawkmoth *M. sexta* has been extensively used as a model to study the role of the ubiquitin system in apoptosis. During the preeclosion period there is a dramatic increase in the expression of proteasome subunits (both 20S and 26S), which correlates with programmed cell death of intersegmental muscles [25]. Accumulation of ubiquitin conjugates is accompanied by induction of enzymes of the E1-E2-E3 cascade [26]. Proteasomes isolated from condemned intersegmental muscles show a ninefold increase in proteinase activity compared with proteasomes from precommitment muscles, an eightfold increase in the absolute amounts of proteasome protein and the appearence of four new subunits that were not detected in the precommitment muscles [27]. A molting hormone agonist that prevents the occurrence of apoptosis in intersegmental muscles also prevents the normally occurring rise in the level of ATPase subunits of the 26S proteasome [28].

Proteasome changes during apoptosis

Before apoptosis is triggered in lung tumor cells, proteasomes are present in the nuclei as well as in the cytoplasm. Upon increased chromatin condensation, nuclear proteasomes are found predominantly surrounding the chromatin, whereas the chromatin itself remains devoid of staining. Proteasomes are clearly detectable in the apoptotic bodies and cytoplasmic vesicles at the time when immunocytochemical reactivity for cytokeratins and lamins has diminished to a large extent [29]. In apoptotic granulosa cells proteasomes migrate to the apoptotic blebs [30]. During dexamethasone-induced apoptosis of thymocytes, cell extracts show a decrease in proteasome chymotrypsin-like activity which correlates with the degree of apoptosis observed [9], while on the contrary, in apoptotic lymphocytes from AIDS patients, there is an activation of the ATP and ubiquitin-dependent pathway of protein degradation [8]. The mRNA of RN3 subunit of the proteasome is upregulated during 1,25-dihydroxyvitamin D3-induced apoptosis of C6.9 rat glioma cells [31].

Changes in the ubiquitinating machinery during apoptosis

A species of the E2 enzyme, responsible for ubiquitination and degradation of topoisomerase II, is activated or induced during the latent phase of E1A-induced apoptosis of the human epidermoid carcinoma-derived cell line MA1 [32, 33]. The lack of a putative 32-kDa E2 enzyme is probably the major cause of the dominant mouse mutation Fused toes (Ft), which is characterized by partial syndactyly of the limbs and thymic hyperplasia. Both morphological abnormalities were shown to be related to impaired regulation of programmed cell death [34].

During apoptosis caspases cleave Nedd4, a ubiquitin-protein ligase containing multiple WW domains and a calcium/lipid binding domain [35]. A giant membrane-associated E2 enzyme BRUCE (BIR repeat containing ubiquitin-conjugating enzyme) bears a baculovirus

inhibitor of apoptosis repeat (BIR) motif, which up to now has been exclusively found in apoptosis inhibitors of the IAP-related protein family(inhibitors of apoptosis) [36].

Interactions of proteasomes and ubiquitinating enzymes with components of the apoptotic machinery

There are also numerous interactions of proteins involved in the transduction of proapoptotic stimuli and components of the ubiquitin-proteasome pathway; however, their significance is unclear. A protein related to a proteasomal subunit binds to the intracellular domain of the TNF-α (tumor necrosis factor α) receptor 1 (TNFR-1) upstream to its death domain [37]. p97 non-ATP-ase subunit of the 19S cap of the human 26S proteasome is very similar to TNFR-1-associated protein (TRAP)-2 and 55.11, both of which were identified as binding proteins of the cytoplasmic domain of TNFR-1 by yeast two-hybrid screening. This finding suggests that the 26S proteasome might serve as a mediator molecule in the TNF signaling pathway in cells [38].

UBC-FAP (ubiquitin conjugating enzyme Fas-associated protein, HsUbc9) is a human ubiquitin-conjugating enzyme found to associate with Fas at the interface between the death domain and the membrane-proximal region. A single amino acid substitution in the death domain of Fas that abolishes Fas-mediated apoptosis also abolished Fas association with UBC-FAP, suggesting that UBC-FAP may play a role in Fas signal transduction [39, 40].

SUMO-1 (small ubiquitin-like modifier, sentrin), a ubiquitin-like protein, interacts with the signal-competent forms of Fas or TNFR-1 death domains, to the extent that when overexpressed it provides protection against both anti-Fas and TNFα-induced cell death [41]. However, in other cell lines its overexpression is by itself a cause of apoptosis [42]. Another ubiquitin-like protein, DAP-1 (death associated protein 1), also interacts with the cytoplasmic death domain of TNF-R1. Overexpression of DAP-1 causes apoptosis of the TNF-α-sensitive L929 fibroblast cell line, as well as of the TNFα-resistant osteosarcoma cell line U2OS. Furthermore, the dominant negative FADD (Fas-associated death domain protein) blocks the cell death induced by DAP-1. This suggests a role for DAP-1 in mediating TNF-α-induced cell death signaling pathways, presumably through the recruitment of FADD death effector [42]. FAF1 (Fas associated factor 1), a Fas-associated protein that potentiates apoptosis, was found to contain sequences similar to ubiquitin [40]. Probably a dominant negative deletion mutant of the avian FAF homologue qFAF causes apoptosis of transfected cells [43].

Myc is an oncoprotein involved in apoptosis. Treatments that cause apoptosis in most systems greatly lower c-*myc* expression, whereas in some others, paradoxically, c-*myc* overexpression accompanied with a withdrawal of growth factors accelerated cell death [44]. Proteasome inhibitors block Myc degradation with the accumulation of Myc-ubiquitin conjugates. Under basal conditions Myc proteins can be conjugated by two pairs of E2s and E3s. In the presence of HPV 16 (human papilloma virus) E6 protein, a third pair, E2-F1 and E6-AP mediate conjugation of Myc by means of a mechanism that appears to be similar to that involved in the targeting of p53. In HPV16 infected cells E6-mediated targeting of Myc for degradation prevents Myc-induced apoptosis and thus ensures maintenance of viral infection [45].

p53 and the proteasome

p53 is the 'guardian of genome integrity'. Upon activation in response to cellular stress, DNA damage, pRb disruption or oncogene expression, p53 induces gene products involved in cell cycle arrest (e.g. $p21^{cip1}$) and apoptosis (e.g. Bax), which leads either to genome repair or to the elimination of a potentially cancerous cell. The stability (and hence level) of p53 protein is normally regulated by its interaction with Mdm-2, which targets p53 for degradation by ubiquitin-mediated events. Since Mdm-2 is itself regulated by p53, loss of function of p53 leads to lack of Mdm-2 and thus to p53 protein accumulation. This provides a mechanistic explanation for the observation that p53 accumulation is associated with neoplasia. In HPV-16/18 infected cells, the viral E6 protein associates with a cellular E3 enzyme called E6-AP, forming a complex with p53, which induces its ubiquitination and degradation by proteasomes [46, 47]. Activation of the tumor suppressor p53 by stress and damage stimuli often correlates with induction of Jun N-terminal kinase (JNK). JNK association with p53 plays an important role in p53 stability [48].

In experimental systems using in vitro transformed primary cells, there is a direct correlation between the presence of p53 and apoptosis. For example, E1A/*ras*-transformed primary mouse fibroblasts are less viable, and undergo apoptosis following genotoxic stress if they contain a wild-type p53. In comparison, similarly transformed cells which are p53-deficient are more viable and do not undergo apoptosis under these conditions [49]. Apoptosis induced by proteasome inhibitors in Rat-1 and PC12 cells is accompanied by a rapid accumulation of p53, and p53-inducible gene products $p21^{cip1}$ and Mdm-2. Apoptosis in this model is inhibited by expression of dominant negative p53, whereas overexpression of wild-type p53 was sufficient to induce apoptosis [50].

Proteasome inhibitors prevented the degradation of the p53-response proteins, Bax and Mdm-2 as well as $p21^{cip1}$ in HeLa cells, and also increased expression of these three proteins in cells that lack p53, showing that stabilization of the p53 response proteins is not due to increased levels of p53 itself. Ubiquitin-dependent protein degradation influences the turnover of downstream targets of p53, suggesting therefore that the proteasome plays a role in regulating apoptosis and cell cycle arrest in response to p53 [51]. BAG-1 (Bcl-2-binding protein with anti-cell death activity gene), a ubiquitin-like Hsp70/Hsc70-regulating protein, is a negative regulator of Siah-1A (p53-inducible human homologue of *Drosophila* seven in absentia), since cotransfection of wild-type BAG-1 with Siah-1A abolished the cell cycle arrest. BAG-1 functions downstream of p53-induced gene expression to inhibit p53-mediated suppression of cell growth, presumably by suppressing the actions of Siah-1A. Siah-1A may be therefore an important mediator of p53-dependent cell cycle arrest, which is directly inhibited by BAG-1 [52].

Proteasome inhibitors as inducers of apoptosis

Numerous proteasome inhibitors are available, among them peptide derivatives (PSI, LLnLal, MG115, MG132 etc.) and the antibiotic lactacystin. Two novel antiproteasomal antibiotics have been recently described – epoxomicin and eponemycin. They also induce apoptosis in several cell lines (Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N., Crews, C. M. (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. PNAS 99, 10403-8 and [109]). Albeit many of those inhibitors are claimed to be more or less specific and inhibitors of other proteases are used as controls, caution should be used when considering these studies [53, 54].

The first report on the proapoptotic action of a proteasome inhibitor came from the studies of lactacystin effects on human monoblastic U937 cells [55]. This initial study was followed by several others.

ZLLLal induced apoptosis accompanied with p53 accululation in MOLT-4 and L5178Y cells, whereas ZLLal, a specific inhibitor of calpain, did not. This suggests that inhibition of proteasome induces p53-dependent apoptosis and that proteasomes can somehow protect cells from apoptosis [56]. RVC lymphoma cells exposed to proteasome inhibitors arrested at the G2/M phase followed by apoptosis [19]. Exposure of Ewing's sarcoma cells to lactacystin resulted in accumulation of ubiquitinated proteins and activation of Bcl-2-sensitive apoptotic pathways [20]. PSI induced a cell cycle block at the G2/M transition followed by a wave of apoptosis in HeLa cells [57], whereas a double G1/S and G2/M block and massive apoptosis as induced PSI in mouse L1210 leukemia cells [58]. A dipeptidyl inhibitor of the proteasome called CEP1612-induced apoptosis in various cancer cell lines tested in a p53-independent manner associated with accumulation of the cyclin-dependent kinase inhibitors $p21^{cip1}$ and $p27^{kip1}$ [59].

Exposure of rodent fibroblasts and human lymphoblasts in culture to Z-LLFal resulted in the induction of apoptosis in a rapid, dose-dependent fashion. Fibroblasts transformed with c-*ras* and c-*myc*, lymphoblasts transformed by c-*myc* alone, and a Burkitt's lymphoma (BL) cell line that overexpresses c-*myc* were up to 40-fold more susceptible to Z-LLFal-induced apoptosis than were either primary rodent fibroblasts or immortalized nontransformed human lymphoblasts, respectively [60].

Recently it has been reported that nitric oxide, which is known to induce apoptosis in many cell types [61], acts most probably through inhibition of the proteasome. Both exposure of cells to *S*-nitrosoglutathione and stimulation of endogenous nitric oxide production by lipopolysaccharide/interferon-γ treatment resulted in inhibition of proteasome activity as measured in vitro. Moreover, chemically diverse nitric oxide donors interfered with proteasome-mediated degradation of polyubiquitinated p53 in vitro. These data imply that nitric oxide- induced apoptosis and accumulation of p53 are, at least in part, mediated by inhibition of the proteasome. Proteasome inhibitors may therefore mimic this action of nitric oxide [62]

Proteasome inhibitors prevent apoptosis

Specific inhibitors of proteasomes block cell death induced by ionizing radiation, glucocorticoids or phorbol ester in thymocytes [63]. At low doses, lactacystin inhibited the activation-induced cell death of T cell hybridoma DO.11.10 cultured in anti-CD3-coated wells, which correlated with inhibition of IκBα degradation, translocation of the NFκB (p50/RelA) into the nucleus, and activation-induced expression of Fas and FasL. However, lactacystin failed to inhibit the killing of DO.11.10 by FasL-expressing allo-specific cytotoxic effector cells. These observations strongly suggest a direct link between the proteasome-dependent degradation of IκBα and the activation-induced cell death that occurs through activation of the FasL gene and upregulation of the Fas gene [64].

Proteasome inhibitors prevented Fos degradation, which usually precedes apoptosis and apoptosis itself in WEHI7.2 cells. Stable transfection with a mutant c-*fos* that is not degraded by the proteasome also inhibited apoptosis. Moreover, overexpression of *bcl*-2 blocked

Fos degradation and inhibited apoptosis. These results indicate that proteasome-mediated Fos degradation is an early step in apoptosis. Fos may have a protective action that is eliminated by proteasome-mediated degradation and preserved by Bcl-2 [65].

Proteasome inhibitors prolonged survival of NGF-deprived sympathetic neurons, which demonstrates that the proteasome is a key regulator of neuronal apoptosis and that, within this process, it is involved upstream of caspase activation. This order of events was confirmed in macrophages where lactacystin inhibited the proteolytic activation of procaspases and the subsequent generation of active interleukin-1β [11]. However, proteasome inhibitors did not prevent apoptosis of sympathetic neurons cultured in the presence of NGF, which overexpressed the proapoptotic proteins Bax and Bak [66]. Moreover, in a recent report it was shown that inhibitors such as MG-115, MG-132 and clasto-lactacystin β-lactone even induce neuronal apoptosis [67].

The effect of different proteasome inibitors in preventing etoposide-apoptosis of rat thymocytes was related to their potency of inhibition. Etoposide caused a rapid p53 accumulation that was not inhibited by proteasome inhibitors, which are also strong inducers of p53. Proteasome inhibitors were also weak activators of caspase activity, suggesting that the same mechanism, i.e. the blocking of proteasome function, both triggers apoptosis and inhibits the effect of etoposide [68]. Proteasome inhibitors also prevented all manifestations of thymocyte apoptosis induced by the glucocorticoid receptor agonist dexamethasone or by the topoisomerase II inhibitor etoposide [69].

TNF-α, NFκB activation and the proteasome system

Since IκBα has been shown to be degraded by the proteasome, proteasome inhibition is the favorite tool to prevent NFκB activation after various stimuli, including TNF-α [70]. This cytokine activates at leasts two different transduction pathways: one which involves caspase activation and apoptosis, and a second which involves NFκB activation and prevents apoptosis [71]. This idea is supported by various experimental models.

Simultaneous treatment of U937 lymphoid cells with TNF-α and proteasome inhibitors increased the apoptotic response and induced a further increase of caspase activity compared with TNF-α alone. These inhibitors were used at low concnetration, which by itself did not increase caspase activity [72].

Proteasome inhibition by lactacystin sensitizes chronic lymphocytic leukemia cells [73] to apoptosis by TNF-α treatment. Treatment of leukemic cells with proteasome inhibitors [74] increased sensitivity to apoptosis induced by TNF-α and doxorubicin, whereas in cells that are resistant toward induction of apoptosis by these stimuli, proteasome inhibition or antagonization of NFκB activity by a nondegradable IκBα partially restored sensitivity to apoptosis [75].

However, there are some reports contradicting the hypothesis of NFκB mediation of the effects of proteasome inhibitors. E.g., MG132 did not exert any effect on TNF-α-dependent apoptosis of U937 and 293 kidney human cells. Pretreatment of these cells with MG132 even reduced JNK-dependent apoptosis caused by heat shock or ethanol [76]. Also, B16F10 melanoma and L1210 mouse leukemia cell lines did not show an enhancement of PSI effects by TNF-α [58, 77].

Possible mechanisms of proteasome involvement in apoptosis

Several possible mechanisms of proteasome involvement in apoptosis could be deduced, based on the degradation of certain short-lived, key substrates. Many potential substrates have already been discussed, among them p53, Bax, Fos, $p21^{cip1}$ and so on; however, none of them appears to have a crucial role in any of the experimental models of apoptosis induced or prevented by proteasome inhibitors [56, 78, 79].

The proteasome-mediated step(s) that promotes apoptosis is most probably located upstream from mitochondrial changes and caspase activation, since proteasome inhibitors prevent the early disruption of the mitochondrial transmembrane potential $\Delta\psi_m$, which precedes caspase activation, exposure of phosphatidylserine and nuclear DNA fragmentation. In contrast, stabilization of $\Delta\psi_m$ or inhibition of caspases does not prevent the activation of proteasomes after the action of apoptotic stimuli [69]. This has been confirmed in two glioma cell lines in which apoptosis induced by lactacystin and LLnLal is independent from mitochondria and p53. Apoptosis in those cells is associated with upregulation of unidentified short-lived proteins. Once caspase-3 is activated, it causes neither cytochrome *c* release nor $\Delta\psi_m$ disruption during the execution phase of apoptosis [80]. The existence of such short-lived proteins has been confirmed by a study of a temperature-sensitive defect in the ubiquitin-activating enzyme E1, which induced apoptosis independent of the activation of caspase-3 and -6. Caspase inhibitors were not capable of blocking this type of cell death, whereas *bcl*-2 overexpression effectively protected cells from apoptosis. Short-lived proteins may serve the role of caspase-independent effectors of apoptosis and attractive targets of the death-protective action of *bcl*-2 [81]. However, high expression of functional Bcl-2 protein in prostate carcinoma cells failed to signal protection against cell

death induction by proteasome inhibitors [82]. It is possible that this was due to Bcl-2 proteolysis, as shown in the case of MO7e cells, where proteasome inhibitors induce apoptosis with caspase-3 activation, which in turn induces Bcl-2 cleavage [83].

In human endothelial cells, Bcl-2 is specifically degraded after stimulation with TNF-α in a process that is inhibited by particular proteasome inhibitors. In addition, the mutation of the potential ubiquitin-acceptor amino acids of Bcl-2 provides protection against TNF-α and staurosporine-induced degradation in vitro and in vivo. Moreover, mimicking phosphorylation of the putative mitogen-activated protein (MAP) kinase sites of the Bcl-2 protein (Thr 56, Thr 74 and Ser 87) abolishes its degradation, suggesting a link between the MAP kinase pathway to the proteasome pathway. Finally, inhibition of Bcl-2 degradation either by suppressing ubiquitin-dependent proteasomal degradation or by mimicking continuous phosphorylation of the putative MAP kinase sites in the Bcl-2 protein confers resistance against induction of apoptosis. Thus, the degradation of Bcl-2 may unleash the inhibitory function of Bcl-2 over the apoptosome complex and may thereby amplify the activation of the caspase cascade [84].

The relation of the proteasome system with the caspase cascade is far more complex. Sometimes, proteasomes degrade a caspase or its processing enzyme, and proteasome inhibition therefore enhances caspase activity [72], whereas in other cases proteasomes themselves display a caspase-like activity [85]. Proteasome inhibition in MO7e human myeloid progenitor cells leads to apoptosis, which is mediated independently by caspases and unknown proteases, inhibited by *N*-tosyl-L-phenylalanine chloromethyl ketone. Thus, not only caspases but also other protease cascades are downstream from the proteasome [86].

A possible target of proteasome-mediated degradation during thymocyte apoptosis could be ornithine decarboxylase, which is known to be degraded by the proteasome without previous ubiquitination [87]. Polyamine levels rapidly decrease in thymocytes undergoing apoptosis, where prevention of apoptosis with proteasome inhibitors also preserves the polyamine levels [88].

JNK and heat shock protein (Hsp)72 is a tempting pair of possible targets of proteasome activity regulating apoptosis. JNK induces at least certain types of apoptosis, whereas Hsp72 prevents it. An antiapoptotic action of otherwise proapoptotic proteasome inhibitors could be revealed by a short incubation of cells with MG132 followed by its withdrawal. Under these conditions Hsp72 accumulated in cells and caused suppression of JNK activation in response to certain stresses. Accordingly, pretreatment with MG132 reduced JNK-dependent apoptosis caused by heat shock or ethanol. Thus proteasome inhibitors simultaneously activate the proapoptotic JNK and induce the antiapoptotic Hsp72. Proteasome inhibitors restore endogenous expression of Hsp72 in aged cells, which leads to suppression of JNK activation by a heat shock and restoration of thermotolerance manifested in a lower rate of apoptosis. The balance between these opposite actions therefore determines the fate of the cell. However, not all types of apoptosis are regulated by this mechanism, since proteasome inhibitors are unable to block JNK-independent apoptosis induced by TNF-α. [76, 89].

In another cases the decision to be or not to be of the cell could be mediated by the ubiquitin-proteasome degradation of the Myc protein, which in some systems is proapoptotic, whereas in other it induces apoptosis [44, 45]. K562 cells, like virtually every case of chronic myelogenous leukemia, are characterized by the presence of the chimeric oncogene *bcr-abl*. Treatment with proteasome inhibitors results, surprisingly, in reduced Bcr-Abl protein expression which occurs several hours before the onset of apoptotic execution, whereas the levels of Bcr and Abl remain unaltered. Reduced Bcr-Abl expression is reflected in significantly attenuated Bcr-Abl-mediated protein tyrosine phosphorylation [90]. In human LNCaP prostate carcinoma cell proteasome inhibitors induced apoptosis accompanied by upregulation of PAR-4 (prostate apoptosis response- 4, a putative proapoptotic effector protein), stabilization of Jun and modest downregulation of Bcl-XL [82].

It has been also suggested that proteasome-mediated apoptosis relies on the induction of the CDK inhibitor $p27^{kip1}$ [91], which is degraded by the ubiquitin pathway [92]. In addition to its role as a CDK inhibitor, p27 is a putative tumor suppressor gene, regulator of drug resistance in solid tumors and promoter of apoptosis [93]. Aggressive tumors may result from the selection of a clone or clones that lack p27 due to increased proteasome-mediated degradation [94].

Concluding remarks

Proteasomes in apoptosis behave like expert diplomats, changing alliances depending on actual circumstances, sometimes protecting the cell from the execution while in other situations contributing to cell death. What is their true role? When a cell faces apoptotic stimuli, are they killers or saviors? Evidence allows no clear answer to that question. Recent papers place proteasomes at the top of the apoptotic machinery, upstream of mitochondrial changes and caspase activation [69, 80, 86]. Such a position in a biochemical pathway usually implies a decision-making step; thus it is possible that the ubiquitin-proteasome system somehow integrates the various stimuli for or against the apoptotic process, and generates a decision which is executed by proteolysis of

certain key protein(s). A perfect example of this model is that proteasome inhibitors activate JNK, which initiates an apoptotic program, and simultaneously they induce Hsp72, which suppresses JNK-dependent apoptosis. A balance between these two effects may define the fate of cells exposed to the inhibitors [76]. Nevertheless, in some cells no evidence was found for activation of the JNK stress kinase pathway during apoptosis induced by proteasome inhibitors [82].

When does proteasome inhibition induce apoptosis and when does it prevent it? Logically, higher concentrations of proteasome inhibitors should be more damaging to cells than lower concentrations, and lower concentrations, while not totally impairing cellular proteolysis may be enough to induce upregulation of key proteins, which could be either inducers or inhibitors of apoptosis. A recent report shows, that whereas at high concentration MG132 and lactacystin do induce apoptosis, at low concentration, when apoptosis is induced in only a fraction of cells, they are able to prevent apoptosis induced by stronger stimuli, such as Sindbis virus exposition [12]. However, generalization of this result is challenged by the logical view, that proapoptotic proteins are generally short-lived compared with generally long-lived antiapoptotic proteins, and therefore that even after transient proteasome inhibition the levels and activities of proapoptotic proteins are much more likely to increase than those of antiapoptotic factors [95].

The sensitivity of each cell to apoptosis can also differ depending on its metabolic activity and cell cycle stage. Drexler proposed a model in which proteasome inhibition prevents apoptosis in quiescent cells but favors it in cycling cells [5]. For example, lactacystin repressed mitogen-induced T cell proliferation. Probably as a result of a blockage of cell cycle progress, cycling, but not resting, T cells underwent apoptosis when treated with lactacystin [96]. Moreover, transformed fibroblasts were much more sensitive to the apoptogenic action of proteasome inhibitors than their wild counterparts [60]. Retinoic acid-induced differentiation of L1210 leukemia cells makes them less susceptible to the proapoptotic action of PSI [10]. Lactacystin activated the apoptotic pathway in chronic lymphocytic leukemia cells, at doses which had no effect in normal cells, where significantly higher concentrations were required [97].

However, there are examples of quiescent cells being killed by proteasome inhibitors [66, 67], whereas some cycling cells survive in their presence [98] and even develop resistance by induction of proteases which replace the proteasome [99, 100]. It remains to be tested whether in cells resistant to the proapoptotic action of proteasome inhibitors there is a constitutively high expression of those proteases. In rat thymocytes the same proteasome inhibitors weakly induced apoptosis by themselves, whereas they prevented apoptosis induced by etoposide [68]. Moreover, many of the inhibitor studies could be questioned, based on the poor specificity of these drugs [53, 54]. Many of the substances used as proteasome inhibitors (e.g. LLnL) are even stronger inhibitors of calpain than of proteasomes. The calcium-dependent protease calpain is required for apoptosis in several mouse models of cell death. E.g., calphostin C-induced apoptosis of U937 human promonocytic leukemia cells is partly calpain-mediated, but does not require protein degradation through the ubiquitin/proteasome pathway [98], whereas potentiation of calpain activity by depleting its endogenous inhibitor, calpastatin, is sufficient to cause apoptosis of neutrophils [101].

What might be the signal upstream of proteasome activity? It is tempting to speculate that it could be nitric oxide [62], which mediates apoptosis induced by Fas, TNF-α and interferon-γ [102].

It has been reported that after oxidative stress proteasomes are poly-ADP-rybosylated, which leads to their increased proteolytic activity. This rybosylation is performed by PARP (poly-ADP-rybose polymerase), the very same enzyme whose cleavage is used as a common indicator of apoptosis [103]. Many if not all apoptogenic effects of nitric oxide can be mediated by inhibition of the proteasome [62], but nitric oxide also activates PARP [104]. It follows that PARP should rybosylate proteasomes, which in turn should lead to their activation. Nitric oxide thus appears to induce two quite opposite effects on proteasomes, and it is possible that the protective effects of nitric oxide in certain types of apoptosis [105, 106] are mediated by proteasome activation. Activation of caspases induces PARP cleavage [107], which in turn prevents proteasome activation.

The exact role of proteasomes in apoptosis remains to be established pending detailed molecular studies, including identification of key regulator proteins which are degraded by proteasomes during apoptosis. Nevertheless, in some cases proteasome inhibitors do prevent apoptosis, and their proapoptotic action is also well documented in the case of many cancer cell lines.

It has been already shown with nude mice that proteasome inhibitors can be effective antitumor drugs in the case of the Burkitt's lymphoma [60] and PC-3 prostate carcinoma [108]. In the latter case, the treatment has been carried out with a new proteasome inhibitor, a highly specific boronic acid derivative, PS-341, which is already being tested in clinical trials of terminally ill cancer patients [108]. In both nude mice cancer models, antitumor activity correlated with the induction of apoptosis. Curiously, no serious adverse effects have been reported.

Mice bearing the syngeneic C-26 colon carcinoma can be successfully treated with the proteasome inhibitor

PSI. However, there is no evidence of increased tumor apoptosis in vivo, despite the induction of apoptosis in vitro, and the mechanism of action is dependent on the inhibition of angiogenesis [T. Stoklosa et al., unpublished results]. Similar conclusions were drawn from the finding that the antitumor and antiangiogenic agent dihydroeponemycin is a bona fide proteasome inhibitor [109]. It is possible that proteasome inhibitors will be used as anticancer drugs before their exact mechanism of action, including their relationship to the apoptotic process, is known [110].

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