Intracellular Protein Degradation and Its Therapeutic Implications

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Lysosomal and ubiquitin (Ub)-mediated protein degradation are two mechanisms for intracellular protein catabolism. The latter is further divided into Ub-proteasome and Ub-aggresome pathways. Recent advances have both defined the mechanisms of protein degradation and identified opportunities for therapeutic applications.

Lysosomal Protein Degradation

Lysosomes are produced in Golgi apparatus for degradation of ingested products. For example, bacteria taken up by phagocytosis are enclosed in vacuoles, which then fuse with vesicles containing lysosomal enzymes (primary lysosomes). As the pH becomes more acidic, enzymes become activated and the vacuole becomes a secondary lysosome degrading the bacterium. Lysosomes also play a key role in destroying old organelles within the cell, allowing them to be replenished. This process is known as autophagy and is accomplished in two stages. First, a membrane is donated by the endoplasmic reticulum. This membrane then surrounds the old organelle. Second, a lysosome fuses with this membrane to form an autophagic vacuole. The lysosome then safely releases its enzyme contents into this vacuole, thereby destroying the old organelle. A third function for autophagosomes is the degradation of proteins and other substances taken in by endocytosis. Materials taken up by inward budding of vesicles from the plasma membrane may first be processed in the endosomal compartment and then delivered to the lumen of a lysosome by fusion with a transport vesicle. The lysosomal proteases include many cysteine proteases (cathpsins) as well as aspartate proteases and a zinc protease. These enzymes are specially synthesized for the lysosome in the endoplasmic reticulum and are active only in an acidic environment.

Several lysosomal storage disorders are known. Acid maltase deficiency (lysosomal glycogen storage disease) leads to the accumulation of glycogen in muscle tissue. Tay-Sachs disease, due to deficiency of one of the enzymes breaking down hexosaminidase, results in lipid deposition in neuronal nerve tissue, leading to severe brain damage and nervous degeneration. Adrenoleukodystrophy, a disorder of the peroxisomes, results in the deposition of lipid in the brain, spinal cord, and adrenal glands, causing dementia and adrenal failure. Gaucher’s disease is due to a deficiency of the lysosomal enzyme glucocerebrosidase, which results in liver and spleen enlargement as well as erosion of the long bones, such as the femur. If the disease manifests in infancy, there is also brain damage causing learning disability.

Ub-Mediated Protein Degradation

Ub is a highly conserved small protein composed of 76 amino acids found only in eukaryotic organisms. The COOH terminus of Ub forms an isopeptide bond with the amino group of a lysine side chain of target protein. Attachment of multiple copies of Ub targets the protein for degradation by the large intracellular protease known as the 26S proteasome. Ub is involved in many cell processes. For example, Ub is conjugated to cyclin during the G1 phase of mitosis, thereby regulating the cell cycle. Ub conjugation also modulates DNA repair, embryogenesis, transcription, and apoptosis.

Role of the proteasome in protein catabolism. The 26S proteasome is an ATP-dependent, multifunctional proteolytic complex that differs in many respects from typical proteolytic enzymes. It consists of a proteolytic core, the 20S (720 kDa) proteasome, sandwiched between two 19S (890 kDa) regulatory complexes. These complexes associate together in an ATP-dependent manner. The 19S proteasome regulatory complexes control the access of substrates to the proteolytic core. The 20S proteasome, a multicatalytic protease, forms a hollow cylinder composed of four stacked rings. Each outer ring is composed of seven different α-subunits and each inner ring is composed of seven distinct β-subunits. Moreover, each β-ring contains caspase-like, trypsin-like, and chymotrypsin-like proteolytically active sites. The 20S proteasome degrades oligonucleotide and protein substrates by endoproteolytic cleavage.

The Ub-proteasome pathway is the major nonslysosomal proteolytic system in the cytosol and nucleus of all eukaryotic cells. It triggers degradation of proteins involved in cell cycle progression, apoptosis, nuclear factor-kB activation, inflammation, as well as immune surveillance. It also degrades mutant, damaged, and misfolded proteins. Degradation of proteins via the Ub-proteasome pathway is a multistep process. First, free Ub is activated in an ATP-dependent manner by Ub-activating enzyme (E1), thereby forming a complex with Ub. Second, Ub is transferred to one of many distinct Ub-conjugating enzymes (E2), which directly ubiquitinate substrate proteins. Third, E3 enzymes, Ub-ligases functioning in concert with E2 enzymes, attach the small Ub moiety to lysine residue of acceptor proteins. The ubiquitinated protein is then shuttled to the proteasome for degradation.
**Role of aggresomes in protein catabolism.** Perinuclear inclusions termed “aggresomes” form in the setting of stress associated with misfolded or mutated proteins. They were first reported in 1998 by Kopito and colleagues (1, 2), who described the appearance of microtubule-associated inclusion bodies in cells overexpressing the pathogenic allele of the cystic fibrosis transmembrane conductance receptor. Subsequently, aggresomes were reported due to overexpression of presenilin1 (1), parkin (3), peripheral myelin protein PMP22 (4), influenza virus nucleoprotein (5), a chimera of green fluorescent protein and the membrane transport protein p115 (6), and notably amyloidogenic light chains (7). These complexes undergo active, retrograde transport to the microtubule-organizing center, mediated in part by a cytoplasmic protein deacetylase, histone deacetylase 6 (HDAC6; ref. 8). HDAC6 is required for aggresome formation associated with ubiquitinated protein stress and cell viability in this context. HDAC6 binds ubiquitinated proteins through a zinc finger domain and interacts with the dynein motor complex through another discrete binding motif. Importantly, chemical and genetic observations have shown that the enzymatic function of HDAC6 is required for aggresome formation (Fig. 1; ref. 8). The precise mechanisms of downstream protein catabolism following aggresome formation are presently being examined. A recent study shows that aggresomal inclusions fuse with autophagosomal vacuoles and that autophagosome delivery to the aggresome is mediated by HDAC6 (9).

**Therapeutic Implications**

Proteasome inhibition was first validated as a treatment strategy in multiple myeloma. Bortezomib (pyrazylcarbonyl-Phe-Leu-boronate; PS-341, Velcade) represents a class of peptide boronate proteasome inhibitors that inhibits the chymotryptic activity of the 26S proteasome (10, 11). Our in vitro studies have confirmed that bortezomib induces apoptosis in conventional drug (dexamethasone, melphalan, and doxorubicin) resistant multiple myeloma cell lines, as

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**Fig. 1.** Possible ubiquitinated protein catabolism in multiple myeloma cells. Misfolded proteins become polyubiquitinated and are normally degraded by proteasomes. However, misfolded proteins can escape degradation due to abnormal or pathologic conditions and form toxic aggregates. These misfolded and aggregated proteins are recognized and bound by HDAC6 through the presence of polyubiquitin chains. This allows for the loading of polyubiquitinated misfolded protein cargo onto the dynein motor complex by HDAC6. The polyubiquitinated cargo-HDAC6-dynein motor complex then travels to the aggresome, where the misfolded and aggregated proteins are processed and degraded, clearing the cell of cytotoxic protein aggregates.
well as freshly isolated patient multiple myeloma cells by: activation of c-Jun NH2-terminal kinase; activation of caspase-8, caspase-9, caspase-3, and poly(ADP-ribose) polymerase cleavage (12–14). Bortezomib also down-regulates expression of adhesion molecules on multiple myeloma cells and bone marrow stromal cells and related binding, blocks constitutive and multiple myeloma cell adhesion-induced nuclear factor kappa-B–dependent cytokine secretion in bone marrow stromal cells (15), and inhibits angiogenesis. Moreover, bortezomib inhibits DNA repair by cleavage of DNA-dependent protein kinase catalytic subunit (14); importantly, treatment of multiple myeloma cell lines resistant to DNA-damaging agents with those agents to which they are resistant, followed 12 to 24 hours later with sublethal doses of bortezomib (16), can inhibit repair of DNA damage and thereby restore drug sensitivity. Bortezomib also induces caspase-dependent cleavage of gp130 (CD130), thereby abrogating interleukin-6-mediated downstream extracellular signal-regulated kinases/mitogen-activated protein kinase, Janus kinase 2/signal transducers and activators of transcription 3, and phosphatidylinositol-3 kinase/Akt signaling pathways, which mediate growth, survival, and drug resistance, respectively, of multiple myeloma cell, in the bone marrow milieu (17). Finally, bortezomib also inhibits human multiple myeloma cell growth, decreases tumor-associated angiogenesis, and prolongs host survival in models of human multiple myeloma in severe combined immunodeficient mice (18).

These preclinical studies have translated to the bedside. Phase I trials in man establishing its safety profile and early evidence of anti–multiple myeloma activity, along with preclinical in vitro and in vivo studies demonstrating that bortezomib can overcome drug resistance and induce apoptosis of multiple myeloma cells in the bone marrow milieu, were completed in 2000 (19). In 2001, a phase II trial of bortezomib treatment of 202 patients with refractory relapsed multiple myeloma showed 35% responses, including 10% complete and near complete responses; response duration of 12 months and survival of 17 months versus the expected 6 to 9 months; and clinical benefit (20). Based on these results, bortezomib was approved by the Food and Drug Administration for treatment of relapsed refractory multiple myeloma in May 2003, with bench to bedside translation and approval in 3 years. In 2005, Food and Drug Administration approval was extended to patients with relapsed multiple myeloma, based on an international multicenter phase III trial comparing bortezomib versus dexamethasone, which showed statistically significant prolongation in time to progression and survival in the bortezomib-treated cohort (21). Clinical phase I/II trials are now combining this agent with conventional (dexamethasone, DNA-damaging agents) as well as novel [lenalidomide (22–26), hsp90 inhibitors (27), and p38MAPK inhibitors (28)] therapies for both patients with relapsed multiple myeloma and those earlier in the disease course. In spite of this remarkable progress, two thirds of patients with relapsed refractory multiple myeloma do not respond to bortezomib, and those with initially responsive disease acquire resistance.

Multiple myeloma is also an ideal system for studying the potential therapeutic utility of targeting the aggresome pathway of protein degradation. We have shown that the proteasome inhibitor bortezomib triggers significant antitumor activity in multiple myeloma both in preclinical models and in patients with relapsed refractory disease. As noted above, studies have shown that unfolded and misfolded ubiquitinated proteins are degraded not only by proteasomes, but also aggresomes, dependent on HDAC6 activity. We, therefore, hypothesized that inhibition of both mechanisms of protein catabolism could induce accumulation of ubiquitinated proteins followed by significant cell stress and cytotoxicity in multiple myeloma cells (Fig. 1). To prove this hypothesis, we used bortezomib and tubacin to inhibit the proteasome and HDAC6 (29), respectively. Tubacin specifically triggers acetylation of α-tubulin as a result of HDAC6 inhibition in a dose- and time-dependent fashion. It induces cytotoxicity in multiple myeloma cells mediated via caspase-dependent apoptosis; in contrast, no toxicity is observed in normal peripheral blood mononuclear cells. Tubacin inhibits the interaction of HDAC6 with dynein, induces marked accumulation of ubiquitinated proteins, and synergistically augments bortezomib-induced cytotoxicity via c-Jun NH2-terminal kinase/caspase activation. Importantly, this combination also induces significant cytotoxicity in plasma cells isolated from multiple myeloma patient bone marrow. Finally, adherence of multiple myeloma cells to bone marrow stromal cells confers growth and resistance to conventional treatments; in contrast, the combination of tubacin and bortezomib triggers toxicity even in adherent multiple myeloma cells (30). Our studies, therefore, show that tubacin combined with bortezomib mediates significant anti–multiple myeloma activity, providing the framework for clinical evaluation of combined therapy to improve patient outcome in multiple myeloma.

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