Testing the ubiquitin–proteasome hypothesis of neurodegeneration in vivo

Abstract

A common histopathological hallmark of most neurodegenerative diseases is the presence of aberrant proteinaceous inclusions inside affected neurons. Because these protein aggregates are detected using antibodies against components of the ubiquitin–proteasome system (UPS), impairment of this machinery for regulated proteolysis has been suggested to be at the root of neurodegeneration. This hypothesis has been difficult to prove in vivo owing to the lack of appropriate tools. The recent report of transgenic mice with ubiquitous expression of a UPS-reporter protein should finally make it possible to test in vivo the role of the UPS in neurodegeneration.

Chains of ubiquitin (a small protein of 76 amino acids) conjugated to a substrate protein specifically target that protein for degradation by the proteasome, a huge multi-subunit protein complex found in all eukaryotic cells. This ubiquitin–proteasome system (UPS) is responsible for the clearance from cells of most soluble proteins and, particularly, of key short-lived regulatory proteins and damaged or misfolded proteins. The first animal model with expression of a reporter protein that allows the detection of UPS impairments has been recently reported. Detecting impairment of the UPS has been a major challenge owing to the complexity of this machinery for regulated proteolysis that requires the coordinated action of many enzymes and protein complexes. These include the E1 ubiquitin-activating enzyme, 20–40 different E2 conjugating enzymes, and hundreds of different E3 ubiquitin ligases. The E2 conjugating enzymes transfer one molecule of activated ubiquitin to a specific E3 ubiquitin ligase that, finally, binds the ubiquitin molecule to a lysine residue in a given substrate protein [3]. Additional ubiquitin molecules are attached to the first one, thus generating the polyubiquitin chain that is recognized by the 19S regulatory complexes located at one or both sides of the 20S proteolytic core of the proteasome. The 19S complexes unfold the polyubiquitinated substrate protein and facilitate its entrance into the 20S proteasome, where it is degraded into small peptides. Polyubiquitin chains are cleaved from the substrate protein and subsequently recycled into monomeric ubiquitin by different deubiquitinating enzymes (DUBs). Alterations in this complex system have been suggested to play a role in different pathological conditions such as cancer, neurodegenerative diseases and muscle-wasting syndromes.

Impairment of the UPS has been postulated to be at the root of neurodegenerative diseases

The notion that alterations in the UPS might play a role in neurodegenerative diseases arises from the observation that in most of these diseases aberrant proteinaceous deposits can be detected inside the affected neurons using anti-ubiquitin and anti-proteasome antibodies. Such deposits include neurofibrillary tangles in Alzheimer's disease, Lewy bodies in Parkinson's disease, neurofilament-containing and superoxide dismutase (SOD1)-containing inclusions in amyotrophic lateral sclerosis, and polyglutamine-containing aggregates in Huntington's disease and other CAG triplet repeat disorders. The UPS-impairment hypothesis is further supported in the case of Parkinson's disease by the finding that mutations in the ubiquitin ligase parkin and the enzyme with combined ubiquitin ligase and deubiquitinating activity (UCH-L1) are the cause of two familial forms of the disease.

It has been suggested that aggregate-prone proteins, such as tau, α -synuclein or mutant huntingtin, are susceptible to adopting an aberrant self-assembling conformation that can lead to the formation of aggregates. Chaperones and the UPS counteract this phenomenon but, if the aggregate is finally built-up, it can sequester different components of the UPS, thus reducing UPS activity. Increased aggregation would lead to a further decline in UPS function, thus leading to a positive feedback mechanism of progression. In the end, this would result in inefficient removal of key regulatory proteins and the accumulation of other abnormal proteins that would cause cellular dysregulation and, eventually, cell death.

Limitations of the available tools to study UPS function

This compelling and unifying hypothesis for the molecular mechanism of neurodegeneration has been difficult to test. Many studies have approached this issue by assaying the proteolysis of small fluorogenic substrates specific for each of the three catalytic activities of the 20S proteasome, in homogenates from postmortem human brain tissue from affected individuals, from mouse models, and from cell models (e.g. cell lines transfected or transduced with the mutant forms of the aggregate-prone proteins). A drawback of exploring UPS activity by assaying proteolysis of these small fluorogenic substrates is that they are degraded by the 20S proteasome in a ubiquitination-independent manner. Therefore, these assays can detect alterations in the catalytic activity of the 20S proteasome but will fail to detect alterations at any other level of the UPS, such as availability of free ubiquitin, polyubiquitination, recognition by the 19S proteasome, and/or unfolding and presentation to the 20S proteasome. Furthermore, it is possible that UPS impairment happens in a very limited number of neurons. In such case, the enzymatic assay might not be able to detect the UPS impairment, owing to a dilution effect of the affected neurons in the context of the tissue homogenate.

Furthermore, exploring the catalytic activity of the 20S proteasomes with fluorogenic substrates has additional basic and practical limitations because 20S proteasomes in a tissue homogenate can be in either a latent form or an activated form, depending on whether detergents or hydrophobic peptides are present during the procedure and depending on the content of proteasome activators such as the 19S and PA28 complexes in each tissue. Finally, studies reporting changes in 20S proteasome catalytic activity in brain homogenates often lack measurement of total proteasome content, which is key for the correct interpretation of the results.

A more integrated analysis of UPS function has been accomplished in cell models transfected to express reporter proteins (e.g. the green-fluorescent protein, GFP) tagged for efficient ubiquitination and subsequent degradation by the proteasome. These proteins have an extremely short half-life but accumulate when cells are exposed to proteasome inhibitors. This approach has revealed UPS impairment in cell models of cystic fibrosis, retinitis pigmentosa and Parkinson's disease, although contradictory results have been obtained in cell models of polyglutamine diseases.

Despite the wealth of results from cell models of neurodegenerative diseases with reporter proteasome substrates, it is key to validate and expand this knowledge *in vivo* in the available mouse models of neurodegenerative diseases. This is important because the level of the pathogenic protein can be extremely high in transfected or transduced cells. By contrast, in the mouse models the levels of toxic proteins are adjusted to a maximum compatible with the normal development of the animals but leading to progression to a relevant phenotype during adult life.

This could be the reason why enzymatic activity assays with small fluorogenic substrates revealed decreased 20S proteasome activity in extracts from transfected and transduced cells but no inhibition was detected in adult mice with overt phenotype and an extensive load of polyglutamine aggregates. Similarly, transfection or transduction of mutant huntingtin into primary cultured neurons results in cell death, whereas primary neuronal cultures from mouse models do not show decreased viability. Accordingly, no loss of striatal neurons is detected at early stages of phenotype progression in mouse models, despite obvious striatal atrophy and/or motor deficits.

UPS-reporter mice

Because excellent mouse models have been previously generated for most neurodegenerative diseases, the recent generation of a transgenic mouse model for *in vivo* monitoring the status of the UPS finally offers the opportunity to address the postulated UPS impairment in neurodegenerative diseases without the limitations of the biochemical and cellular models. For the generation of these mice, Lindsten et al. employed their previously generated reporter substrate UbG76V-GFP. The UbG76V-GFP reporter carries a ubiquitin fusion degradation (UFD) signal, consisting of an N-terminal-linked ubiquitin molecule that serves as acceptor for polyubiquitin chains, through the canonical Lys48 and the less common Lys29 in the N-terminal-fused ubiquitin. The G76V substitution prevents removal of the N-terminal-fused ubiquitin by cellular DUB enzymes, leading to efficient ubiquitination and proteasomal degradation of the UbG76V-GFP reporter.

Lindsten et al. generated transgenic mice expressing UbG76V-GFP under control of the cytomegalovirus immediate-early enhancer and the chicken β -actin promoter. Expression was confirmed by RT-PCR in all analyzed tissues and northern blots revealed highest levels of transcripts in muscle, heart, testis and brain. Despite the transcription of the UbG76V-GFP transgene in all analyzed tissues, no fluorescence was observed in any tissue, confirming the instability of the UbG76V-GFP protein *in vivo*, similar to what had been shown in cell systems.

To evaluate the functionality of the UbG76V-GFP reporter *in vivo*, Lindsten et al. [2] administered intraperitoneally different proteasome inhibitors, such as epoxomicin and MG-262. A low dose of MG-262 resulted in accumulation of the UbG76V-GFP reporter in a subpopulation of liver cells, and a higher and toxic dose resulted in detectable fluorescence in most liver cells and also in small intestine, pancreas, kidney, lung and spleen. Although no fluorescent cells were detected in the brain, heart or skeletal muscle, primary cultured neurons derived from the UbG76V-GFP mice became fluorescent after exposure to 0.5 μ M of the specific proteasome inhibitor epoxomicin. More importantly, infection of primary neurons from UbG76V-GFP mice with a lentiviral vector encoding UBB+1 (an aberrant form of ubiquitin that accumulates in affected neurons of patients with Alzheimer's disease) also resulted in accumulation of the reporter protein. This demonstrates that the reporter system is sensitive enough to detect UPS impairments in neurons, and in particular those caused by mechanisms relevant to the etiology of neurodegenerative diseases.

It should be noted, however, that despite the great advantages of the UbG76V-GFP-reporter mice in detecting UPS impairments *in vivo*, they still have some limitations that might preclude the detection of an existing UPS impairment. This is because proteins with UFD signals, such as the UbG76V-GFP-reporter protein expressed in these mice, are ubiquitinated by specific subsets of E2 conjugating enzymes (e.g. Ubc4 and Ubc5) and E3 ubiquitin ligases (e.g. Ufd2). This reporter system might therefore fail to detect UPS impairments if other E2 and E3 enzymes are involved, or if the defect is in an early recognition step, such as that of recognition by a chaperone before degradation.

It is conceivable that different transgenic lines of UPS-reporter mice will be generated in the near future with expression of different reporter proteins (each requiring different combinations of E2 and E3 enzymes for their degradation). The availability of a full array of these reporter mouse lines will maximize the chance of detecting UPS impairments in the available mouse models of neurodegenerative diseases.

Concluding remarks

In summary, the recently reported generation of transgenic mice with constitutive and ubiquitous expression of the UbG76V-GFP-reporter protein will allow the hypothesis of impaired UPS activity in neurodegenerative diseases to be explored in vivo. By breeding with the currently available mouse models of neurodegenerative diseases, in the immediate future it should be possible to know whether this reporter protein accumulates in the brains of symptomatic mice. Furthermore, if this generally accepted hypothesis is confirmed, it should be possible to identify the specific neuronal subpopulations in which the UPS impairment takes place.

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