Clearance of Mutant Proteins as a Therapeutic Target in Neurodegenerative Diseases

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Accumulation and aggregation of disease-causing proteins is a hallmark of several neurodegenerative disorders such as Parkinson, Alzheimer, and Huntington diseases. One of the main goals of research in neurodegenerative disorders has been to improve clearance of these accumulated proteins. Using the example of Huntington disease, I discuss strategies to selectively activate cellular degradation machinery to improve clearance of the mutant protein and to identify therapeutic targets for the treatment of Huntington disease and related neurodegenerative disorders.

Huntington disease (HD) is an autosomal dominant disease that afflicts approximately 1 in 10,000 people. Huntington disease commonly presents in adulthood with personality changes, cognitive (eg, dementia, difficulties with executive function) and psychiatric disturbances (eg, depression, psychosis, obsessive-compulsive disorder, substance abuse, paranoia), and abnormal movements. The movement disorder is typically chorea, but dystonia, myoclonus, athetosis, and rigidity may also be seen. Huntington disease remains fatal because no effective treatments can cure the disease or slow its progression.

Huntington disease is caused by a mutation in the huntingtin gene that encodes a protein of 3144 amino acids with a molecular weight of about 350 kDa. The mutation is an expansion of the CAG trinucleotide repeat coding for a polyglutamine tract in the Htt protein. Healthy individuals have CAG repeat lengths of approximately 7 to 34, whereas in HD patients the CAG repeat is expanded and inversely correlated with the age of disease onset. Repeat lengths of more than 40 glutamines invariably produce HD, and very high glutamine repeats (eg, >80) can lead to onset of HD in children or young adults. The huntingtin protein is expressed throughout the body, but HD is not simply a striatal disease, evidenced by degeneration of many other brain regions in later stages of the disease.

Huntington disease belongs to a family of polyglutamine disorders that are caused by the expansion of the CAG repeat within the coding region of various genes; the expansion results in expanded polyglutamine tracts in the respective proteins. In addition to expanded polyglutamine repeats, these diseases share other features such as adult onset, progressive neurodegeneration, an inverse correlation of numbers of repeats with the age of onset, and the presence of polyglutamine protein–containing inclusions.

Levels of Mutant Huntingtin Correlate with Neurotoxicity

The accumulation and aggregation of misfolded proteins is a pathological hallmark of most age-related neurodegenerative disorders, including HD. Significant evidence suggests that levels of mutant Htt strongly correlate with severity of phenotypes. This is best demonstrated through a conditional mouse model, in which elimination of mutant Htt expression resulted in reversal of the pathological phenotype. Similar correlations have been observed in other mouse models of polyQ-mediated neurodegeneration, including a conditional model for spinocerebellar ataxia type 1. Such accumulation of mutant Htt presumably leads to alterations in multiple cellular pathways such as gene transcription, energy metabolism, axonal transport, synaptic trans-
mission, and vesicle release. Although the presence of intracellular aggregates composed of mutant Htt protein in the brains of patients with HD suggests that mutant Htt does not get properly cleared from the brain, the relative role of aggregated or nonaggregated Htt in disease pathogenesis remains unclear. Recent studies indicate that soluble forms of mutant Htt may represent a toxic species of Htt. Numerous other theories have been proposed to explain the mechanism of pathogenesis in HD and other polyglutamine disorders. Although it remains unclear which of these mechanisms have the greatest effect on HD pathogenesis, it is well established that the expression level of mutant Htt is a critical predictor of degeneration. Selective clearance of mutant Htt is therefore expected to provide the therapeutic benefit in HD.

**MECHANISMS OF MUTANT HUNTINGTIN CLEARANCE**

Although the degradation mechanism of mutant Htt protein has not been clearly established, the 2 main cellular degradation pathways—the ubiquitin proteasome system and the autophagic-lysosomal pathway—have been implicated.

Proteasomal-mediated degradation is an adenosine triphosphate–dependent process that relies on ubiquitination of the substrate followed by unfolding and translocation into the narrow proteasomal pore. It remains controversial whether the ubiquitin proteasome system, which is responsible for selectively degrading damaged or misfolded proteins, can also degrade mutant Htt. For example, it has been suggested that the ubiquitin proteasome system is inhibited by abnormal polyglutamine stretches. Although proteasomal inhibitors lead to the formation of Htt aggregates and ubiquitin has been found in Htt-containing inclusion bodies, other studies suggested that polyglutamine tracts cannot be degraded by the proteasome. Aberrant proteolytic cleavage by caspase 6 to release a polyQ-containing 586 amino acid N-terminal fragment of mutant Htt has also been linked to HD pathogenesis. Numerous other theories have been proposed to explain the mechanism of pathogenesis in HD and other polyglutamine disorders. Although it remains unclear which of these mechanisms have the greatest effect on HD pathogenesis, it is well established that the expression level of mutant Htt is a critical predictor of degeneration. Selective clearance of mutant Htt is therefore expected to provide the therapeutic benefit in HD.

Autophagy consists of several processes by which the lysosome acquires cytosolic cargo, with the following 3 types of autophagy being discerned in the literature: (1) macroautophagy, characterized by the formation of a crescent–shaped structure (the phagophore) that expands to form the double-membrane autophagosome, capable of fusion with the lysosome; (2) microautophagy, in which lysosomes invaginate and directly sequester cytosolic components; and (3) chaperone-mediated autophagy, which involves translocation of unfolded proteins across the lysosomal membrane. Because the main focus of this review is on the process of macroautophagy, I will hereinafter refer to it throughout the text as simply autophagy. In autophagy, cytoplasmic content or an organelle is sequestered in double-membrane vesicles called autophagosomes. Autophagosomes then fuse to lysosomes for degradation of substrates. This process is controlled by highly conserved autophagy (ATG) genes. Core ATG proteins, including beclin, Atg 7, and microtubule-associated protein light chain 3 protein (LC3), are essential for the formation of autophagosomes. Beclin is involved in the initial autophagosome formation through a phosphatidylinositol 3-0H kinase complex, whereas Atg 7 and LC3 are involved in autophagic vesicle elongation through a ubiquitin–like conjugation pathway.

Recent studies have identified LC3-interacting proteins, such as p62, that play a role in the autophagic clearance of protein aggregates. A multifunctional protein, p62 has been shown to bind ubiquitin and LC3, possibly providing a molecular shuttle for misfolded aggregated proteins to promote their clearance by autophagy. This protein is known to localize with Htt aggregates and can modulate Htt-induced cell death. Therefore, p62 may act as an adapter autophagy protein by linking toxic substrates to core autophagic machinery and facilitating the clearance of these substrates by the autophagic-lysosomal pathway.

Initial findings demonstrating the accumulation of autophagic vesicles in the brains of patients with HD have led to the hypothesis that autophagy contributes to the pathogenesis of HD. Subsequent studies have suggested that Htt aggregates may be cleared by autophagy. In addition, administration of small molecules that promote autophagy has improved phenotypes in cell and animal models of HD. Studies using pharmacological inhibitors or enhancers of autophagy have suggested that mutant Htt is a substrate for autophagic degradation. For example, inhibition of autophagosome formation with 3-methyladenine or inhibition of autophagosome-lysosome fusion with bafilomycin A1 inhibited the clearance of mutant Htt, induced the formation of inclusions, and increased cell death. Conversely, enhancing autophagy with rapamycin accelerated mutant Htt clearance and improved cell viability. The levels of wild-type Htt were not affected by these compounds, suggesting that autophagy plays a specific role in the clearance of aggregate-prone Htt.

Although autophagosomes can sequester cytosolic material nonspecifically, for example, as a response to starvation, there is evidence in yeast for selective autophagic degradation of various cellular structures, including protein aggregates, mitochondria, and microbes. On the other hand, targeted and selective degradation of misfolded proteins by autophagy is not well understood in mammalian
systems and represents an important avenue of research. Our recent findings suggest that selective targeting of mutant Htt to autophagosomes for degradation can be achieved by acetylation of the mutant protein.25

**ACETYLATION OF MUTANT HUNTINGTIN PROMOTES ITS CLEARANCE**

Although autophagy appears to play an important role in neurodegeneration, current approaches to modulate autophagy result in a global and nonspecific activation of autophagy that could have deleterious consequences in neurons. To address selectivity of the degradation process, we examined whether modification of the target protein itself may promote its clearance without disrupting other cellular pathways.25 To this end, the study found that selective clearance can be achieved by posttranslational modification of the mutant Htt by acetylation at lysine residue 444 (K444). Increased acetylation at K444 facilitates trafficking of mutant Htt into autophagosomes, significantly improves clearance of the mutant protein by autophagy, and reverses the toxic effects of mutant huntingtin in vitro and in vivo (Figure 1). These studies suggest a critical role for acetylation in the control of regulated clearance of mutant huntingtin and provide an exciting therapeutic opportunity.

In eukaryotic cells, acetylation is among the most common covalent modifications and ranks similar to the important master switch phosphorylation.26,27 The correlation between histone acetylation and increased transcription has been known for many years, but acetylases are now being identified to modify a number of non-histone proteins.27 Acetylation can affect many cellular functions, including protein–protein interactions, microtubule dynamics, splicing, messenger RNA function, protein localization, metabolism, protein stability, and aging.27 Acetylation of lysines can block ubiquitination at the same residue, thereby preventing proteasomal degradation. Acetylation of proteins can also promote enhanced degradation. For example, acetylation of globin transcription factor 1, pRB, and hypoxia-inducible factor 1 was reported to induce their degradation.27 Therefore, modification of specific lysine residues by covalent bonding with an acetyl group modulates the biological functions of many proteins and protein complexes.

Protein acetylation is very dynamic and is maintained by the following 2 classes of functionally antagonistic enzymes: the protein acetylases (HATs) and the deacetylases (HDACs).28 Because histones were the first identified targets of HDACs, these enzymes were termed histone deacetylases. The large and continuously growing number of nonhistone targets undoubtedly demonstrates that histones are only some of the many substrates of HATs and HDACs. The superfamily of HDACs consists of 3 main subtypes: classes I, IIa, IIb, and IV and the structurally distinct class III.27,28 Classes I and II HDACs include the zinc29–dependent HDACs, which share significant structural homology, especially within the highly conserved catalytic domains. By contrast, the class III HDACs, or sirtuins, are structurally different from other HDACs and functionally different in their dependence on nicotinamide adenine dinucleotide+ to accomplish catalytic reactions, which include HDAC and mono-adenosine diphosphate–transferase activities.

Unlike HDACs, HATs are more diverse in structure and function.27,28 In mammals, more than 30 HATs display distinct substrate specificities for histones and non-histone proteins. Many HATs possess an evolutionarily conserved protein module specifically recognizing acetyllysines, that is, the bromodomain that targets chromatin-associated proteins to acetylated histones. The most intensively studied HATs are CREB-binding protein (CBP) and p300, which both contain a bromodomain and are often found within the same complexes.

Recent studies demonstrate that mutant Htt interacts directly with the histone acetyltransferase (HAT) domain of CBP.29 Previous studies also demonstrated that depletion of CBP enhanced toxicity, whereas overexpression of CBP suppressed toxicity by mutant Htt.30,31 Data from a study by Jeong et al25 showed that acetylation of mutant Htt by CBP leads to neuroprotection by improving clearance of the mutant protein. Although CBP is not sufficiently specific as a therapeutic agent, acetylation of mutant Htt represents a novel pathway that should allow for development of more selective compounds (eg, HDAC inhibitors) to specifically promote acetylation and clearance of mutant Htt in HD.

**THERAPEUTIC IMPLICATIONS**

Significant evidence suggests that levels of mutant Htt strongly correlate with severity of phenotypes.4,6 On the other hand, loss of wild-type function not only contributes to HD pathologic changes,32 but also leads to neurodegeneration and motor dysfunction in its own right.27 Because mutant Htt performs some normal functions of Htt,1 a complete or drastic knockdown of the mutant protein is expected to have a negative effect on the long-term course of HD. Therefore, from the standpoint of therapy for hu-
man HD, a moderate decrease of mutant Htt levels (but not of the wild-type Htt) would be expected to delay disease onset and progression. The data suggest that selective clearance of the mutant Htt protein can be achieved by hyperacetylation of the mutant protein with HDAC inhibitors.25

Baseline acetylation of only mutant but not wild-type Htt has been detected in cultured cells and in mouse or human HD brain. Because we observe relatively rapid clearance of wild-type Htt under baseline conditions when compared with the mutant protein, wild-type protein may also be acetylated but degraded too rapidly to permit detection. The accumulation of mutant Htt in patients with HD, despite acetylation, is clearly an indication that even this attempt by the neuron is not sufficient to evade disease onset; however, because the data in mice and cultured neurons indicate that the acetylation-resistant form of mutant Htt accumulates even more, it can be concluded that some benefit arises from baseline acetylation (Figure 2). Moreover, the data suggest that, by increasing the rate of acetylation at K444 by CBP or HDAC inhibitors, we can enhance degradation of mutant Htt that results in neuroprotection, suggesting that acetylation of K444 represents a means of regulated clearance of mutant Htt.25

Previous studies have shown HDAC inhibitors to be neuroprotective in various HD models.25 Although the precise mechanism of protection has not been elucidated, it has been attributed to several possible targets, including chromatin remodeling and amelioration of transcriptional dysregulation by inhibition of HDACs.28 Genetic modulation of HDACs in a Drosophila melanogaster model of HD suggested that specific protection is mediated by knockdown of rpd3 (fly orthologue of HDAC1/2) and Sirt1 and Sir2 orthologues.28,29 Inhibition of HDAC also led to improvements of mitochondrial-dependent calcium2 release, the data suggest that, by increasing the rate of acetylation at K444 by HDAC inhibitors, we can enhance degradation of mutant Htt that results in neuroprotection, suggesting that acetylation of K444 represents a means of regulated clearance of mutant Htt.25

Most previous studies using HDAC inhibitors were performed with shorter mutant Htt fragments that did not contain K444, suggesting that the observed mechanisms of action were independent of K444-mediated clearance of mutant Htt. Unlike short fragments that aggregate readily, the longer Htt fragments and full-length Htt used in our study are more resistant to aggregation, a phenomenon often seen with more complete lengths of Htt. However, these shorter fragments, although a useful experimental tool, especially for studying Htt aggregation, have not been identified in human HD. The fragments on which we focus our work constitute a readily identified fragment that is released by caspase-3- and caspase-6-mediated cleavage. Recent data showed that cleavage of mutant Htt at the caspase 6 site (aa586) represents the required step for neurodegeneration by mutant Htt.10 Our results suggest that acetylation of K444 plays a key role in the modulation of toxic effects and clearance of this critical caspase 6 fragment. Furthermore, acetylation of K444 can lead to the degradation of the soluble protein before the formation of visible aggregates. In support of this notion, the enrichment of acetylation of other substrates implicated in HD may be also affected, independently or as a consequence of

Figure 2. Acetylation-resistant mutant Htt accumulates in mouse brains. Brains of wild-type mice were stereotaxically injected with lentiviral vectors expressing acetylatable (Htt571-72Q) or acetylation-resistant (Htt571-72Q-KR) mutant Htt. Although no significant accumulation was noted 4 weeks after injection (compare A and B), a dramatic accumulation of 72Q-KR compared with 72Q Htt was observed 13 weeks after the injection (compare C, E, and G with D, F, and H). The accumulation of Htt 72Q-KR led to increased neurodegeneration as determined by a significant decrease in the mean neuronal volumes of infected neurons.25 Serial sections from at least 10 mice in each group were examined. Representative sections show predominantly cytoplasmic neuronal expression of lentivirus-Htt571-72Q (A, C, E, and G) and cytoplasmic and nuclear expression of lentivirus-Htt571-72Q-KR (B, D, F, and H) (original magnification ×100 [A, B, and E-H] and ×40 [C and D]). Reproduced from Jeong et al25 with permission from Elsevier.
acetylation of mutant Htt. Although we did not explore this issue in detail, the use of acetylation-resistant mutant Htt (444KKR) provided an important control for possible nonspecific effects of hyperacetylation. 

Nevertheless, for therapeutic purposes, it would also be important to identify HDAC inhibitors that primarily acetylate the target of interest such as mutant Htt. To this end, Jeong et al. showed that knockdown of endogenous HDAC1 can enhance acetylation and clearance of mutant Htt, suggesting that HDAC1-specific inhibitors may have an effect on mutant Htt clearance. A recent focus of intensive investigation has been to develop class- and isoform-specific HDAC inhibitors, which has been difficult because of the highly conserved HDAC active site.

In addition, the therapeutic application of HDAC inhibitors for diseases of the central nervous system depends on identification of potent, brain-permeable, and bioavailable small molecules. Nevertheless, great advances have been made toward the development of classes I and II isoform-selective HDAC inhibitors that are less cytotoxic. Although brain permeability remains a major limitation, brain penetration has been demonstrated for the HDAC inhibitors vorinostat, sodium butyrate, phenylbutyrate, MS-275, and valproic acid. At present, it is unclear whether class- or isoform-specific inhibitors will be more efficacious for treatment of HD. However, isoform-specific inhibitors would be anticipated to have fewer adverse effects and thus be better tolerated during long-term or shorter-term administration.

Taken together, these results demonstrate that a direct modification of the disease protein promotes its specific clearance and provides a link between protein acetylation and targeted degradation by autophagy. It will be of interest to examine whether protein acetylation represents a more general regulatory mechanism for selective targeting of proteins for lysosomal degradation.

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