ARTICLE

In vitro evidence for both the nucleus and cytoplasm as subcellular sites of pathogenesis in Huntington’s disease

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ABSTRACT

A unifying feature of the CAG expansion diseases is the formation of intracellular aggregates composed of the mutant polyglutamine-expanded protein. Despite the presence of aggregates in affected patients, the precise relationship between aggregates and disease pathogenesis is unresolved. Results from in vivo and in vitro studies of mutant huntingtin have lead to the hypothesis that nuclear localization of aggregates is critical for the pathology of Huntington’s disease (HD). We tested this hypothesis using a 293T cell culture model system that compared the frequency and toxicity of cytoplasmic and nuclear huntingtin aggregates. We first assessed the mode of nuclear transport of N-terminal fragments of huntingtin, and show that the predicted endogenous NLS is not functional, providing data in support of passive nuclear transport. This result suggests that proteolysis is a necessary step for nuclear entry of huntingtin. Additionally, insertion of nuclear import or export sequences into huntingtin fragments containing 548 or 151 amino acids was used to reverse the normal localization of these proteins. Changing the subcellular localization of the fragments did not influence their total aggregate frequency. There were also no significant differences in toxicity associated with the presence of nuclear compared with cytoplasmic aggregates. The findings of nuclear and cytoplasmic aggregates in affected brains, together with these in vitro data, support the nucleus and cytosol as subcellular sites for pathogenesis in HD.

INTRODUCTION

The expansion of a polymorphic CAG tract encoding glutamine is the causative mutation in eight human neurodegenerative diseases, including Huntington’s disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA), and spinocerebellar ataxia (SCA) types 1, 2, 3, 6 and 7 (1,2). Each disease affects specific populations of neurons and results in a characteristic clinical phenotype. Additionally, the mutant genes responsible for these diseases have no sequence similarity except for the CAG tracts. However, there may be a common step in the pathogenic pathways of the diseases that involve novel properties of the expanded CAG tract.

Several reports in the past year have described intracellular protein inclusions, or aggregates, within and outside the nuclei of cells expressing proteins with expanded polyglutamine tracts (reviewed in 1,3,4). Several lines of evidence from patient samples, transgenic mice and cell culture models suggest that the aggregates are associated with the pathology of CAG expansion diseases. First, the inclusions are only observed in the brains of individuals carrying the disease allele, and are present predominantly in regions and neuronal populations affected by the disease (5–12). Secondly, neuronal inclusions have also been observed in mice transgenic for genes with expanded CAG tracts, which develop inclusions prior to onset of neurological symptoms (10,13,14). Thirdly, increasing frequency of aggregates is associated with increasing toxicity in in vitro models of HD, DRPLA and SBMA (9,15,16). Furthermore, decreasing the frequency of aggregates in vitro results in reduced toxicity (9,15). However, even though these data suggest a causal relationship, they cannot differentiate between aggregates as being crucial to pathogenesis, from being markers of pathology.

Recent attention has focused on the nucleus as the primary site of pathogenic changes in polyglutamine expansion diseases. One line of evidence for nuclear involvement is the exclusive nuclear localization of aggregates in post-mortem brains in SCA-1 (10),
SCA-3 (8), SCA-7 (17) and DRPLA (9,11). Intranuclear aggregates were also observed in mice transgenic for an HPRT gene containing an expanded polyglutamine stretch, and in mice expressing exon 1 of the HD gene (13,14). Unusual nuclear morphology, including irregular indentation of the nuclear membrane, increased pore density and chromatin condensation have also been described in transgenic mice (10,13) and patient brains (18,19).

Aggregates were found to be exclusively nuclear in one study of adult and juvenile HD patients (11). However, other studies using different antibodies have identified both intranuclear and cytosolic accumulations of huntingtin in brain tissue of HD patients (5,6). In brains of severely affected juvenile HD patients, huntingtin-containing inclusions were identified within the nuclei of neurons in the cortex and striatum (5). Adult HD patients also displayed extranuclear accumulations of huntingtin in dystrophic neurites and perikarya (5,6). Neurons containing cytoplasmic accumulation were more frequent in adult patients than neurons with nuclear inclusions (5), indicating that cytoplasmic aggregates can also be cytotoxic. Therefore, whether the specific subcellular localization of the huntingtin aggregates is a contributing factor to the pathology in HD is not clear.

In vitro studies have demonstrated that nuclear localization of huntingtin aggregates is influenced by the length of the protein (16,20). Small huntingtin proteins are both nuclear and cytoplasmic whereas larger proteins are only cytoplasmic, suggesting that passive diffusion plays a role in intracellular localization. Proteolytic cleavage would therefore be necessary to reduce the large full-length huntingtin protein to a fragment that is capable of diffusion through the nuclear pores (<60 kDa) (21). However, we also identified a basic amino acid rich sequence within the N-terminus of huntingtin with significant homology to a functional NLS (16), which suggested that huntingtin may also enter the nucleus by active transport. In order to determine the mode of nuclear transport, we assessed the ability of the predicted NLS to transport mutant huntingtin into the nucleus.

The predominance of nuclear aggregates has led us and others to postulate the nucleus as the site of pathology in HD (1,3–5,8,13). This would suggest that a key event in the pathogenesis of HD is the translocation of truncated mutant huntingtin to the nucleus, where it exerts its toxic effect. An unanswered question is whether these aggregates may be similarly toxic outside the nucleus. Additionally, toxicity could be mediated by cell-specific vulnerability to mutant huntingtin and aggregate formation in the nucleus, or by interaction with specific nuclear proteins.

In this study we have designed experiments to specifically investigate the influence of the site of aggregation on toxicity. To do this, we have altered the localization of huntingtin fragments by the addition of nuclear export and nuclear localization sequences. Our results indicate that the aggregates have equivalent toxicity regardless of their location within the cell. Therefore, both the nucleus and cytoplasm represent sites of toxicity of huntingtin.

RESULTS

N-terminal huntingtin fragments enter the nucleus by passive diffusion

To determine whether huntingtin fragments enter the nucleus by passive or active transport, we assessed whether the putative NLS in the N-terminal domain of huntingtin is functional. The 771-128 protein is a huntingtin fragment that includes amino acids 1 to 151, has 128 polyglutamines and contains the predicted NLS at amino acids 90–99 (Fig. 1). The 771-128 cDNA was fused to pyruvate kinase (PK), a large cytoplasmic protein that does not normally enter the nucleus unless attached to an active NLS (22). When expressed in transfected HEK (human embryonic kidney) 293T cells, the majority of the 31 kDa PK–771-128 fusion protein was exclusively cytoplasmic (Fig. 2A; Table 1). In contrast, using anti-c-myc antibody for detection, the large 91 kDa PK–771-128 fusion protein was exclusively cytoplasmic (Fig. 2B). Cytoplasmic localization was also observed for PK–771-128 using the anti-huntingtin antibody MAb 2166 (data not shown). Thus, the NLS in huntingtin is not functional in 293T cells, indicating that huntingtin in vivo has to be proteolytically cleaved to a smaller size, allowing passive diffusion and entry into the nucleus.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Nuclear aggregates (%)</th>
<th>Cytoplasmic aggregates (%)</th>
<th>Total no. cells with aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955-128</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
<td>7.8 ± 2.6</td>
</tr>
<tr>
<td>1955-128-NLS</td>
<td>100 ± 0</td>
<td>0 ± 0</td>
<td>54 ± 1.7</td>
</tr>
<tr>
<td>1955-128-mNLS</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
<td>27 ± 1.5</td>
</tr>
<tr>
<td>771-128</td>
<td>49.2 ± 15.7</td>
<td>50.8 ± 15.7</td>
<td>75.4 ± 10.7</td>
</tr>
<tr>
<td>771-128-NES</td>
<td>2.0 ± 2.0</td>
<td>98.0 ± 2.0</td>
<td>51.3 ± 16.5</td>
</tr>
<tr>
<td>771-128-mNES</td>
<td>51.6 ± 18.0</td>
<td>48.4 ± 18.0</td>
<td>68.4 ± 19.0</td>
</tr>
</tbody>
</table>

*There were no significant differences between the total aggregate frequency between the different 1955 proteins or 771 proteins, except between 1955-128 and the control protein 1955-128-mNLS (P < 0.01).

Altering the location of aggregates formed by the 1955-128 protein does not influence toxicity

To assess whether the subcellular site of aggregation influences toxicity, we altered the localization of aggregates formed by the 1955-128 protein and compared their toxicity in different subcellular locations. The 1955-128 huntingtin fragment includes...
Figure 2. The predicted endogenous NLS in huntingtin is not functional in 293T cells. Huntingtin appears as red stain, the nucleus is counter-stained blue. Nuclear huntingtin stain is pink when the red stain is overlapped with blue. Immunofluorescence of 771-128 (A) using the anti-huntingtin antibody MAb 2166 demonstrates nuclear localization of huntingtin. In contrast, the pyruvate kinase-771-128 fusion protein (B), detected with c-myc, is cytoplasmic.

Figure 3. The localization of huntingtin is altered by the addition of active NLS or NES sequences. Huntingtin, detected by MAb 2166, appears as red stain, the nucleus is counter-stained blue. Nuclear huntingtin stain is pink when the red stain is overlapped with blue. The huntingtin aggregates appear as large clumped masses, easily differentiated from normal diffuse stain. The size of the aggregates varied from cell to cell, but there was no consistent size difference between aggregates formed by the protein products of the three 1955-128 constructs. The aggregates formed by the 1955-128 protein are cytoplasmic (A), those formed by the 1955-128-NLS protein, with an active NLS, are nuclear (B), and those formed by the 1955-128-mNLS control protein, containing a mutant NLS, are cytoplasmic (C). The protein product of the 1955-15 construct is cytoplasmic (D), the 1955-15-NLS protein, with an active NLS, is nuclear (E) and the 1955-15-mNLS control protein, containing a mutant NLS, is cytoplasmic (F).

amino acids 1–548, corresponding in size to the fragment produced by caspase 3 cleavage (23,24), and contains 128 polyglutamines. This fragment has been consistently identified in transfected cells undergoing stress (25), suggesting that it may be a stable fragment produced from full-length huntingtin.

The cytoplasmic perinuclear location of 1955-128 aggregates was changed to a nuclear location by the addition of an NLS. The NLS from the SV40 large T antigen (PKKKRKV) was inserted into the N-terminus of 1955-128, forming 1955-128-NLS (Fig. 1). Immunofluorescence of cells transfected with the 1955-128 constructs was used to quantify their subcellular localization (Table 1). In several cell types and using multiple anti-huntingtin antibodies, the 1955-128 protein is exclusively cytoplasmic (25) (Fig. 3). The 1955-128 protein has a predicted molecular weight of 73 kDa, which is too large to enter the nucleus by passive diffusion. In contrast to 1955-128, 100% of cells expressing 1955-128-NLS protein had nuclear huntingtin stain (Fig. 3), indicating that the ectopic NLS is functional when introduced into huntingtin. The total frequency of aggregates was similar between the 1955-128 and 1955-128-NLS proteins (Table 1). However, the
proportion of aggregates in the nucleus differed between the proteins, with 0% nuclear aggregates for 1955-128 and 100% for 1955-128-NLS, in parallel with the total nuclear stain.

A mutant NLS (PAAAAAV) was also inserted into 1955-128 (forming 1955-128-mNLS), to control for any effect of an introduced peptide on toxicity and aggregate formation. Immunofluorescence on transfected cells showed that the 1955-128-mNLS protein had 0% nuclear stain (Fig. 3). Despite the differences in subcellular localization of 1955-128-mNLS and 1955-128-NLS proteins, there was no significant difference in frequency of aggregates (Table 1).

In the 293T cell model, expression of proteins with an expanded polyglutamine tract results in an increase in susceptibility to apoptotic stress from treatment with a sub-lethal concentration of tamoxifen (15,16; Ellerby et al., submitted for publication). The resultant cell death is quantified by an MTT assay, a standard apoptosis assay (26) that is a sensitive indicator of cell viability. Mock transfected cells and LacZ transfected cells, both treated with tamoxifen, are used as controls. To compare the toxicity of aggregates from the same sized huntingtin proteins in different cellular compartments, 293T cells were transfected with the 1955-128 constructs containing the functional and mutant NLS sequences. As shown in Table 2, there was no significant difference in cell death due to expression of the 1955-128 and 1955-128-NLS proteins (n = 5), indicating that the toxicity of nuclear aggregates was the same as cytoplasmic aggregates. The control, 1955-128-mNLS, which forms cytoplasmic aggregates, also had the same toxicity as 1955-128 (Table 2), indicating that addition of a peptide had no influence on toxicity. Western blotting demonstrated equivalent expression levels for each construct (data not shown). Therefore, aggregates generated by 1955-128 proteins have similar toxicity regardless of their localization.

### Table 2. Importing huntingtin into the nucleus does not change its toxicity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Toxicity relative to LacZ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955-128</td>
<td>71.88 ± 1.36</td>
</tr>
<tr>
<td>1955-128-NLS</td>
<td>72.66 ± 1.37</td>
</tr>
<tr>
<td>1955-128-mNLS</td>
<td>72.70 ± 0.67</td>
</tr>
<tr>
<td>1955-15</td>
<td>92.6 ± 0.91</td>
</tr>
<tr>
<td>1955-15-NLS</td>
<td>92.3 ± 0.53</td>
</tr>
<tr>
<td>1955-15-mNLS</td>
<td>92.0 ± 1.05</td>
</tr>
</tbody>
</table>

Apoptosis assessed at 48 h post-transfection. The 1955-128 proteins are significantly more toxic than the 1955-15 proteins at P < 0.001 (n = 5).

### Table 3. Exporting huntingtin from the nucleus does not change its toxicity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Toxicity relative to LacZ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>771-128</td>
<td>72.13 ± 0.95</td>
</tr>
<tr>
<td>771-128-NES</td>
<td>72.06 ± 1.03</td>
</tr>
<tr>
<td>771-128-mNES</td>
<td>71.81 ± 0.53</td>
</tr>
<tr>
<td>771-15</td>
<td>90.24 ± 0.85</td>
</tr>
<tr>
<td>771-15-NES</td>
<td>90.67 ± 0.99</td>
</tr>
<tr>
<td>771-15-mNES</td>
<td>90.41 ± 0.68</td>
</tr>
</tbody>
</table>

Apoptosis was assessed for the 771 constructs at 36 h post-transfection instead of 48 h to maximize the proportion of 771-128-NES in the cytoplasm. The 771-128 proteins are significantly more toxic than the 771-15 proteins at P < 0.001 (n = 5).

Moving 771-128 aggregates out of the nucleus does not change their toxicity

The 771-128 protein has the highest frequency of aggregates in the nucleus, compared with other huntingtin fragments assessed (16). The 771-128 protein is also extremely toxic to cells in the presence of an apoptotic stress. To further assess the influence of subcellular localization of huntingtin on toxicity, we created a construct that brought the 771-128 protein out of the nucleus, and compared the toxicity of nuclear and cytoplasmic aggregates.

A nine residue nuclear export sequence (LALKLAGLDI) from the CAMP-dependent protein kinase inhibitor was inserted into 771-128, forming 771-128-NES (Fig. 1). Immunofluorescence studies on 293T cells expressing the 771-128 proteins are shown in Figure 4. At 36 h post-transfection the percent of cells expressing 771-128 with nuclear aggregates was 49%. In contrast, the percent of nuclear aggregates of the 771-128-NES protein was <2% (Table 1), indicating that the NES is functional when inserted into huntingtin. The total frequency of aggregates formed by 771-128 and 771-128-NES was not significantly different (Table 1).

At 48 h post-transfection, the proportion of nuclear aggregates was higher for the 771-128-NES protein than at 36 h, most likely because passive diffusion into the nucleus occurred at a greater rate than the energy-dependent active transport out of the nucleus using the NES. A time-course assessment of aggregate formation determined that the highest proportion of cytoplasmic aggregates formed by 771-128-NES occurred at 36 h post-transfection (data not shown). Therefore, toxicity studies for this set of experiments were performed at 36 h post-transfection. Since the analysis of 771-128 was performed at 36 h, while the analysis of the 1955-128 proteins was at 48 h, the aggregate frequency and cell viabilities between these experiments cannot be compared.

To control for addition of a peptide, a 771-128-mNES construct was created with a mutant NES, AAAAAAAGADA. Immunofluorescence studies demonstrated that the proportion of aggregates in the nucleus was 52% for 771-128-mNES, which is similar to the 771-128 protein, but differs substantially from the 771-128-NES protein. The total frequency of aggregates formed by the 771-128-mNES protein was not statistically different to both the 771-128 and 771-128-NES proteins (Table 1).

The toxicity of aggregates formed by the 771-128 proteins in different subcellular locations was compared at 36 h (Table 3). There was no significant difference in toxicity between aggregates formed by 771-128 and 771-128-NES proteins (n = 5). In addition, there was no difference in toxicity between aggregates formed by 771-128 and 771-128-mNES, indicating that addition of a peptide had no effect (n = 5). There was also no difference in toxicity between the 771 proteins at 48 h, although overall toxicity at 48 h was greater than at 36 h due to higher protein expression (data not shown; 16). Western blotting confirmed equivalent expression levels (data not shown). These results show that the 771-128 aggregates in the nucleus were associated with similar levels of toxicity as aggregates outside the nucleus. Therefore, the subcellular localization of 771-128 aggregates does not influence susceptibility to cell death.

The subcellular localization of huntingtin fragments with wild-type polyglutamine tracts does not influence toxicity

In the 293T cell model, huntingtin with longer CAG tracts (128 glutamines) have significantly higher toxicity than shorter tracts (15 glutamines) (16,25). This repeat-length dependence on
Figure 4. The localization of wild-type huntingtin is altered by the addition of active NLS or NES sequences. Huntingtin is shown in red, the nucleus is counter-stained blue, and nuclear huntingtin stain is pink when the red stain is overlapped with blue. The size of the aggregates varied from cell to cell, but there was no consistent size difference between aggregates formed by the three 771-128 proteins. 771-128 protein forms nuclear aggregates (A), 771-128-NES protein, containing an active NES, forms predominantly cytoplasmic aggregates (B) and the 771-128-mNES control protein, containing a mutant NES, forms nuclear aggregates (C). In contrast, 771-15 protein is nuclear (D), 771-15-NES protein, containing an active NES, is cytoplasmic (E), and the 771-15-mNES control protein, containing a mutant NES, is nuclear (F).

To alter the localization of the 1955-15 protein, the SV40 NLS sequence was inserted into 1955-15-NLS (Fig. 1). Immunofluorescence of the 1955-15 and 1955-15-NLS is shown in Figure 3. At 48 h post-transfection, the percent of cells with nuclear stain was 0% for 1955-15 and 100% for 1955-15-NLS (Table 4). The control peptide, encoding a non-functional NLS, was also inserted into 1955-15, forming 1955-15-mNLS. The 1955-15-mNLS protein had 0% nuclear stain (Fig. 3; Table 4). Although the subcellular localizations were obviously different, when tested for toxicity, there was no significant difference between the 1955-15, 1955-15-NLS and 1955-15-mNLS proteins (Table 2). Therefore, for the same sized proteins, the wild-type huntingtin fragments in the cytoplasm have the same susceptibility to cell death as nuclear huntingtin.

Table 4. The proportion of cells with nuclear or cytoplasmic huntingtin is presented as a percent of the total number of cells expressing huntingtin

<table>
<thead>
<tr>
<th>Construct</th>
<th>Nuclear localization (%)</th>
<th>Cytoplasmic localization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955-15</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>1955-15-NLS</td>
<td>100 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1955-15-mNLS</td>
<td>0 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>771-15</td>
<td>76 ± 8.6</td>
<td>24 ± 8.6</td>
</tr>
<tr>
<td>771-15-NES</td>
<td>58 ± 12.5</td>
<td>42 ± 12.5</td>
</tr>
<tr>
<td>771-15-mNES</td>
<td>75 ± 9.2</td>
<td>25.3 ± 9.2</td>
</tr>
</tbody>
</table>

However, the toxicity associated with the 1955-128 proteins was significantly greater than that seen with the 1955-15 proteins (P < 0.001, n = 5) (Table 2). This result demonstrates the potent influence of increasing CAG repeat length on susceptibility to cell death, as shown previously (16,25).

To change the predominantly nuclear 771-15 to a predominantly cytoplasmic protein, the NES sequence was inserted into 771-15, forming 771-15-NES (Fig. 1). At 36 h post-transfection the percent of cells with nuclear stain was 76%. The number of cells with nuclear protein was reduced to 58% for 771-15-NES. The control peptide encoding a non-functional NES was also inserted into 771-15, to create 771-15-mNES. The percent of nuclear stain for 771-15-mNES was 75%, similar to the parental protein 771-15 (Fig. 4) (Table 4). Consistent with the experiments described above, altering the localization of the 771-15 proteins also did not
result in significant differences in toxicity (Table 3). The 771-15 protein, which is predominantly nuclear, had similar toxicity to the 771-15-NES protein, which is predominantly in the cytosol. Furthermore, the 771-15-NES protein had similar toxicity to the product of the control construct, 771-15-mNES, with predominant nuclear localization. Therefore, the subcellular localization of the 771-15 proteins does not influence their toxicity. In addition, there was a significant decrease in cell death associated with expression of the 771-15 proteins compared with the 771-128 proteins ($P < 0.001, n = 5$), as described for the 1955 proteins. Since the assessment of viabilities of the products of the 771-15 constructs was performed at 36 h, and the 1955-15 analysis was at 48 h, the values cannot be compared between experiments.

**DISCUSSION**

**Huntingtin enters the nucleus by passive transport**

An important consideration in studies of the formation of nuclear inclusions in HD is the means by which mutant huntingtin translocates into the nucleus. We identified a sequence motif in the N-terminus of huntingtin that is similar to the NLS of the familial breast cancer BRCA-1 protein (16). Here in 293T cells, we demonstrate that the predicted endogenous NLS in huntingtin is not active, indicating that huntingtin must be reduced in size to a fragment <60 kDa to passively diffuse through the nuclear pores. This data implicates proteolytic cleavage of huntingtin as requirement for nuclear entry, as suggested previously (5). However, our results do not exclude the possibility that specific import factors are present in adult striatum that may facilitate nuclear transport of huntingtin.

**The subcellular localization of huntingtin aggregates does not influence toxicity**

Several lines of evidence have suggested that HD is a disease of the nucleus. Intranuclear inclusions are the predominant markers in affected patients (5,6,11), and mice transgenic for huntingtin exon 1 develop nuclear aggregates prior to the onset of debilitating neurological symptoms (13). Furthermore, increasing toxicity of successively smaller huntingtin fragments in our cell culture model was associated with the formation of nuclear aggregates (16). Increasing toxicity in vitro was also associated with increased aggregate frequency (16). In this study, we have directly addressed the questions of whether the site of huntingtin influences its toxicity, and how huntingtin enters the nucleus.

We altered the subcellular localizations of huntingtin protein fragments with the addition of NLS and NES peptides, while the frequency of aggregates remained equivalent. The cytoplasmic protein 1955-128 was changed to a protein that forms nuclear aggregates. The 771-128 protein, which forms predominantly nuclear aggregates, was altered to form predominantly cytoplasmic aggregates. Our results in this cell culture model indicate that toxicity is not dependent on the subcellular localization of aggregates, but toxicity is associated with the frequency of aggregate formation.

**The cell culture model can mimic in vivo events**

Despite the fact that HD is a disease of selective neuronal death, this 293T in vitro model (25) has been shown previously to recapitulate several features of HD. As observed in vivo, mutant huntingtin and other disease proteins containing expanded polyglutamine tracts form aggregates in vitro, whereas wild-type proteins with a normal tract do not (9,10,15,16,20,27–30). Aggregates formed in vivo and in vitro are frequently ubiquitinated (9,20,27,31; A.S.Hackam, unpublished observations). Furthermore, as observed in post-mortem neocortical tissue (11), increasing polyglutamine length is associated with an increased frequency of aggregates in vitro (25,27,30). More severe grades of HD have higher frequencies of cortical nuclear inclusions (11). Similarly, huntingtin fragments that are more toxic in vitro form higher frequencies of aggregates (16,20). The formation of huntingtin cleavage fragments is observed both in vivo and in vitro (5,25,27). Finally, the selective vulnerability of affected neurons in HD is mimicked by increased susceptibility to apoptotic stress of cultured cells expressing mutant huntingtin (16,20,25). Therefore, it is plausible that these in vitro data, recapitulate the in vivo situation to a significant extent, are relevant, suggesting that nuclear and cytoplasmic aggregates may also have equivalent toxicity in humans.

**Aggregates are not the sole contributors to toxicity**

Intracellular aggregates, regardless of their cellular compartment, are associated with toxicity. Therefore, reducing aggregate frequency remains an important therapeutic target for CAG expansion diseases. At the present time, it is unclear whether aggregates are the primary cause of neurodegeneration, or whether they are formed as an early secondary response to cell injury. The observation that the development of aggregates precedes cell death in vivo and in vitro does not distinguish between aggregate formation as a causal event, or aggregates as a byproduct of other cytotoxic events that lead to death. Aggregates could theoretically even serve a protective role by sequestering toxic polyglutamine containing fragments.

There is increasing evidence that aggregates are not the sole contributors to toxicity. Although the majority of inclusions have been identified in neuronal populations that degenerate during disease progression, several studies have shown that the concordance between nuclear inclusions and neurodegeneration is not absolute (11,12,17,32). There are several examples in which inclusions have been identified in cells not destined to die. Aggregates were present in the dentate nucleus of the cerebellum in HD patients (11), an area that does not frequently exhibit neurodegeneration. Intranuclear inclusions were also identified in SCA-7 (17) and DRPLA (11) patients in regions of the brain not affected by the disease. Ubiquitinylated intranuclear inclusions formed by mutant androgen receptor aggregates were observed in SMB patients in peripheral tissues (12). In addition, mice transgenic for mutant huntingtin exon 1 (13) and a Drosophila model of SCA-3 (32), contained aggregates in regions that do not exhibit cell death. These observations suggest that aggregate formation is associated, but clearly is not sufficient to cause cell death.

There are also reports in which nuclear inclusions were not observed in affected tissues. Neurodegeneration of Purkinje cells in SCA-7 and juvenile HD patients occurred in the absence of aggregates (11,17). Furthermore, there is no apparent correlation of nuclear inclusion frequency with length of the CAG tract and Vonsattel grade in the striatum of HD patients (11).

These observations contradict a role of aggregates as a direct cause of neurodegeneration. However, aggregates may result in deficiencies in neuronal function (1,3,13) and additional events...
may be required to lead to neurodegeneration subsequent to the formation of aggregates. For example, although neurons may initially form aggregates as a result of stress-induced protein cleavage, a particular cellular environment may be required for cytotoxicity, analogous to the tamoxifen-induced stress in our 293T cell model. In neurons, this ‘toxic environment’, possibly resulting from expression of certain glutamate receptors leading to uncontrolled excitotoxicity, may be the stimulus needed for aggregate-containing cells to die. The cells in non-affected tissues that form aggregates could be more resistant to toxicity if they have a higher threshold of injury needed for death. The threshold could be set by the particular repertoire of glutamate receptors, by the cell’s ability to deal with metabolic stress, or by levels of anti-apoptotic factors. Thus, aggregates alone are insufficient for cell death, but selective populations of vulnerable neurons may be more susceptible to a ‘toxic environment’ when their viability is compromised by the presence of aggregates.

**Huntingtin is toxic in both the nucleus and cytoplasm of 293T cells**

Determining the primary site of pathology of the CAG diseases is important for designing therapeutic interventions. Nuclear inclusions are associated with disease in the other CAG diseases studied so far (8–11,17), whereas for HD both nuclear and extranuclear aggregates are seen in vivo and in vitro (5).

The different localization of aggregates in HD compared with other expansion diseases may depend on several factors. First, the size of a protein influences nuclear entry (21). Thus, the ability of the protein to be cleaved into fragments small enough to enter the nucleus is important. Ataxin-3 is small enough to diffuse into the nucleus. The mutant exon 1 fragment in the HD mice forms nuclear inclusions since it is also small enough to diffuse into the nucleus. Only antibodies against N-terminal epitopes recognize nuclear inclusions in HD post-mortem tissue, suggesting proteolytic processing of full-length huntingtin (5,11).

Secondly, the polyglutamine-containing proteins may usually reside in the nucleus as part of its normal function. For example, ataxin-1 (10) is predominantly normally localized in the nucleus of neurons affected in SCA-1. Thus, nuclear entry is not a feature of this disease. In addition, ataxin-3 (8,33), ataxin-7 (34,35), the androgen receptor (12) and atrophin-1 (36) have putative nuclear localization sequences and have been identified in the nucleus. In contrast, we have not found an active NLS in the N-terminus of huntingtin.

Thirdly, there may be inherent differences in the pathogenesis of these diseases due to differences in functional properties or protein partners of the respective proteins. For example, androgen receptor toxicity is influenced by ligand concentration in 293T cells (15), and huntingtin associates with several proteins that have altered interactions with increased polyglutamine length (37–39). Ataxin-1 and ataxin-3 associate with the nuclear matrix (33,40) and may interfere with essential nuclear events as part of their toxicity. Indeed, recent results from ataxin-1 transgenic mouse lines by Klement et al. (41) indicate that nuclear localization of ataxin-1 is critical for SCA-1 pathology. Finally, detection of the localization of a particular protein could vary with tissue preparation and the antisera used.

There is clearly **in vivo** evidence for extranuclear aggregates being toxic (5). While this paper was under review, Sandou et al. (42) presented data that indicated that nuclear localization was required for cytotoxicity of huntingtin in a striatal neuronal line. There are several methodological differences that could account for the discordant conclusions between the present paper and the work of Sandou et al. (42). In this study, we have assessed the total frequency of aggregates, including cytoplasmic aggregates, which allowed direct comparison of the influence of total cellular aggregates on toxicity in 293T cells. Additionally, there are differences in sensitivities and timing of the cell viability assays used. The MTT assay quantifies mitochondrial changes, which are considered as earlier indicators of apoptosis than nuclear morphological changes (43). Mitochondrial markers of apoptosis may record early subtle alterations of cell viability caused by cytoplasmic huntingtin. In addition, further studies are needed to determine whether cell line specific factors may contribute to these differences in findings.

**Considerations for the development of therapeutic strategies**

The results from this study indicate that the differences in toxicity between huntingtin constructs expressed in culture coincide more with aggregate frequency than their site of localization. These aggregates, regardless of their subcellular location, may increase the vulnerability of specific neuronal populations to stress. Alternatively, the aggregates could possibly be involved in pathology by trapping essential nuclear or cytoplasmic proteins, or sequestering huntingtin so that it cannot perform its normal activity or maintain its normal associations.

The data in this manuscript highlight several issues germane to therapeutic strategies for HD. Reducing nuclear transport of huntingtin to eliminate the formation of nuclear aggregates is not likely to be effective for reducing neuronal death in HD since extranuclear aggregates have equivalent toxicity. Instead, decreasing aggregate frequency may be a more appropriate target. Furthermore, these data support initial hypotheses in which cleavage of huntingtin, which contributes to aggregate formation, should be prevented. Indeed, transfection of an uncleavable form of androgen receptor resulted in decreased aggregate formation and reduced toxicity (15). Identifying cellular upstream events leading to aggregate formation, and downstream events resulting from aggregate formation, will be vital in designing additional strategies. It will also be crucial to understand why some cell populations have aggregates but are not dying. Thus, distinguishing the events necessary for aggregate formation and cell death will be essential for our understanding of HD pathogenesis and the identification of targets for intervention.

**MATERIALS AND METHODS**

**cDNA constructs**

The expression constructs containing the first 771 or 1955 nucleotides of huntingtin cDNA with 15 or 128 CAGs (771-15, 771-128, 1955-15 and 1955-128) were created by introduction of a termination codon by site-directed mutagenesis, as described previously (25,44). A huntingtin–pyruvate kinase fusion protein was created using PCR to amplify the 771-128 cDNA (sense primer, 5′-GGTACCCCATGCGACCCCTGGAAAAG-3′; antisense primer, 5′-GAGCTCGGAGCTGAACCTTGGAAAG-3′). The PCR product was ligated into the TA vector (Invitrogen, CA) then subcloned into the C-terminus of the chicken pyruvate kinase cDNA epitope tagged with c-myc, generously provided by Dr Gideon Dreyfuss (University of Pennsylvania) (45).
Insertion of SV40 large T antigen nuclear localization sequence (NLS) (46) and protein kinase inhibitor export sequence (NES) (47) were performed by standard subcloning techniques. Briefly, oligonucleotides containing the NES or NLS sequences, flanked by NheI and EcoRI sites, were annealed together, then ligated into the corresponding sites within the multiple cloning site and the N-terminus of huntingtin. The oligonucleotides also included a Kozak consensus sequence (48) and initiating methionine and the N-terminus of huntingtin. The oligonucleotides also ligated into the corresponding sites within the multiple cloning site ECL (Amersham, Arlington Heights, IL).

To compare the expression levels from the different huntingtin constructs, transfected 293T cells were harvested and lysed in homogenization buffer (20 mM Tris–HCl, 0.3 M sucrose, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM PMSE). Equal amounts of total protein were electrophoresed using standard 7.0% SDS–PAGE gels, transferred onto PVDF membrane and Western blotted using anti-huntingtin antibody BKP1 (39), then detected using ECL (Amersham, Arlington Heights, IL).

**Cell viability assays**

A modified MTT assay was used to measure cell viability (26,49), as described previously (16,25). Transfected cells in 96-well plates were treated at 36 or 48 h post-transfection with a sublethal concentration of tamoxifen (35 μM) for a total of 2 h (50). Mock transfected, vector only and LacZ transfected cells served as controls for transfection-related toxicity. For the modified MTT assay, tamoxifen-treated or untreated cells were incubated for 1 h in a 1:10 dilution of WST-1 reagent (Boehringer Mannheim) and release of formazan from mitochondria was quantified at 450 nm using an ELISA plate reader (Dynatech Laboratories, Chantilly, VA) (26,51). One-way ANOVA and Newman-Keuls test were used for statistical analysis.

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