Expression of Polyglutamine-expanded Huntingtin Activates the SEK1-JNK Pathway and Induces Apoptosis in a Hippocampal Neuronal Cell Line*

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Huntington's disease is one of a growing number of hereditary neurodegenerative disorders caused by expansion of a polyglutamine stretch at the NH₂ terminus of huntingtin. To explore whether polyglutamine-expanded huntingtin induces neuronal toxicity, I examined the expression of the full-length of huntingtin with 16, 48, or 89 polyglutamine repeats in a rat hippocampal neuronal cell (HN33). Expression of mutated huntingtin with 48 or 89 polyglutamine repeats stimulated c-Jun amino-terminal kinases (JNKs) activity and induced apoptotic cell death in HN33 cells while expression of normal huntingtin with 16 polyglutamine repeats had no toxic effect. The JNK activation precedes apoptotic cell death and co-expression of a dominant negative mutant form of stress-signaling kinase (SEK1) nearly completely blocked activation of JNKs and neuronal apoptosis mediated by mutated huntingtin. Taken together, my studies demonstrate that expression of polyglutamine-expanded huntingtin induces neuronal apoptosis via activation of the SEK1-JNK pathway.

Huntington's disease (HD)1 is a progressive neurodegenerative disorder with an autosomal dominant inheritance (1). The genetic defect of the HD gene involves an expansion of a CAG repeat where normal and expanded HD allele sizes range between 6 and 37 and 34 to 121, respectively (1–4). The most common pathologic allele in the HD gene has between 40 and 49 CAG repeats and is clinically manifested by middle age onset with choreiform movement, mental impairment, and cognitive dysfunction due to a selective loss of striatal, cortical, and hippocampal neurons (1–4). Juvenile HD, associated with a longer CAG repeat, is characterized by a severe and diffuse loss of neurons throughout the brains (1–4).

Although the HD gene has been identified for several years, the molecular mechanism by which mutated huntingtin induces neurons to die remains unclear. Many studies have suggested that glutamate-mediated excitotoxicity may play an essential role in the pathogenesis of HD (7). Administration of NMDA receptor agonists such as quinolinic acid induces a behavioral phenotype and neuropathology strikingly similar to that observed in HD patients (8). Apparently, polyglutamine repeat-expanded huntingtin and NMDA receptors may stimulate a common neurotoxic pathway to cause neuronal injury. Therefore, identification of such a neurotoxic pathway may be not only crucial for understanding of the mechanism of neuronal death induced by mutated huntingtin and glutamate, but also for identifying potential drug targets for the treatment of HD as well as other neurodegenerative diseases.

Activation of JNKs has also been implicated in neuronal death induced by a wide range of environmental stress stimuli and glutamate-mediated excitotoxicity. In both primary cultured sympathetic neurons and differentiated pheochromocytoma PC12 cells, deprivation of nerve growth factor leads to the JNK activation and neuronal apoptosis (9, 10). In primary cultured striatal neurons, stimulation of NMDA receptors leads to activation of JNKs and increases of c-Jun and c-Fos expression (11). Intriguingly, gene-targeted knockout of JNK3, an isoform of JNKs that is almost exclusively expressed in the brain, results in a remarkable resistance to neuronal excitotoxicity induced by kainic acid (12). Seizures and hippocampal neuronal apoptosis are prevented, while phosphorylation of c-Jun and the transcription activity of AP-1 are markedly reduced in the JNK3 (−/−) mice (12).

The present study was undertaken to determine whether expression of full-length polyglutamine expanded huntingtin induces neuronal toxicity and to explore the potential molecular mechanism of neuronal death. I found that expression of full-length huntingtin with 48 or 89 polyglutamine repeats resulted in the JNK activation and apoptotic cell death of an immortalized rat hippocampal neuronal cell line (HN33). Co-expression of a dominant negative form of SEK1 nearly completely blocked mutated huntingtin-mediated neuronal apoptosis. Thus, I conclude that polyglutamine-expanded huntingtin, via activation of JNKs mediates neuronal apoptosis.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors for Full-length Huntingtin with 16, 48, or 89 CAG Repeats—The first third of the full-length construct was derived by ligation of IT16LL (bp 932–3018) with three different PCR products (bp 2401–3270, bp 637–1429, and 187–858). A 3027-bp cDNA fragment was lifted from this resulting construct and ligated to corresponding sites in the cDNA clone IT15B (bp 3024–10366). The CAG repeat size in this full-length huntingtin construct, pFL16HD, is 16. PCR products were generated from the genomic DNA of an adult patient with 48 CAG repeats and a juvenile onset case with 89 CAG repeats. These PCR products were ligated to replace the corresponding region in pFL16HD to generate the pFL48HD and pFL89HD with 48 and 89 CAG repeats, respectively. Colony hybridization and PCR were used to identify the 48 and 89 CAG huntingtin clones, and positive clones were verified by DNA sequence analysis.

Cell Culture and Transient Expression of Normal and Polyglutamine-expanded Huntingtin—HN33 cells, cultured in 10-cm plates were initially maintained in DMEM medium supplemented with 10% of fetal bovine serum. After 20–30 passages, cells became differentiated, exhibiting neurite outgrowth. The medium was switched to DMEM/
Mutated Huntingtin Induces Neuronal Apoptosis

RESULTS

To assess whether polyglutamine-expanded huntingtin causes neuronal toxicity, full-length huntingtin expression constructs containing 16, 48, or 89 CAG repeats, respectively, were generated by assembly of a combination of reverse transcription-PCR products from normal and human HD lymphoblast and plasmid cDNA clones IT16L and IT15B (1). To test whether the resulting constructs pFL16HD, pFL48HD, or pFL89HD express different huntingtins, they were transiently transfected into 293 embryonic kidney cells. Expression of either normal or polyglutamine-expanded huntingtin in 293 cells did not generate visible cell toxicity, and the rate of cell proliferation remained unaltered. Transfected cells were lysed 72 h after transfection, and the levels of huntingtin expression were analyzed by immunoblotting using an anti-huntingtin monoclonal antibody, 4C8 (15). As shown in Fig. 1, all three huntingtin constructs constitutively express the huntingtin protein, similar to previous reports (15), mutant proteins migrate slightly slower than normal huntingtin (Fig. 1). Because transfection efficiency is about 40–50% according to β-galactosidase staining (data not shown), the amount of polyglutamine-expanded huntingtin expressed in these transfectants under an optional condition is about three to five times higher than that of endogenous huntingtin in 293 cells (Fig. 1).

Next, I examined whether expression of polyglutamine-expanded huntingtin induces neuronal toxicity. Since hippocampus is one of the brain regions affected in HD (2, 4), I used an immortalized rat hippocampal neuronal cell line, HN33, in my experiments (13). To normalize the expression level in different transfection experiments, 60 μg of plasmid DNA/60-mm plate and the same amount of Lipofectin were used in all experiments, and under this condition, the levels of expression of huntingtin were similar according to huntingtin immunoblotting. Transfection of pcDNA1 (vector) or normal huntingtin constructs constitutively express the huntingtin protein, whereas expression of normal huntingtin slightly suppressed proliferation of HN33 cells (Fig. 2A, middle panel). Expression of mutated huntingtin with 48 or 89 polyglutamine repeats (pFL48HD or pFL89HD), however, clearly induced cell toxicity in HN33 cells. Apoptosis was initially observed between 20 and 24 h after transfection of pFL48HD or pFL89HD. At 48 h after transfection, about ~75% of HN33 cells were apoptotic. As shown in the right panel of Fig. 2A, 24 h after transfection of pFL48HD, a small population of HN33 cells was stained positively by TUNEL, and at 48 h after transfection of pFL89HD, most cells were detached from the slide, and most remaining attached cells exhibited DNA fragmentation. Although transfection efficiency is about 30–40%, according to β-galactosidase staining, about 90–95% of HN33 cells were apoptotic at 72 h after transfection of either pFL48HD or pFL89HD. A similar phenomenon has been observed following injection of the Rac target protein POSH into COS-1 cells, which lead to apoptosis of injected and noninjected neighboring cells (19). Since POSH is also a JNK activator, one possible explanation is that the JNK-activated apoptotic cells may produce and secrete a toxin that contributes to the death of neighboring cells. In addition, scattered neuronal cells in culture may be more vulnerable to neurotoxic stimuli.

Mutated huntingtin with 48 or 89 polyglutamine repeats correlates with middle age and juvenile onset of HD, respectively (5, 6). To examine whether mutated huntingtin with 89 polyglutamine repeats generates earlier and more severe neuronal toxicity than that induced by mutated huntingtin with 48 polyglutamine repeats, I performed a detailed time course of
Mutated Huntingtin Induces Neuronal Apoptosis

HN33 cell survival following transfection with different huntingtin constructs. HN33 cells were transfected with pcDNA1, pFL16HD, pFL48HD, or pFL89HD, and after different times of transfection as indicated in Fig. 2B, cells were fixed and stained. Since most apoptotic cells were detached from the plates, TUNEL-negative cells were counted. Compared with transfection of pcDNA1 (control), transfection with normal huntingtin containing 16 polyglutamine repeats did not significantly reduce the number of TUNEL-negative cells between 12 and 72 h after transfection (Fig. 2B). As expected, neuronal toxicity induced by transfection with mutated huntingtin containing 89 polyglutamine repeats occurred 2–3 h earlier than that mediated by mutated huntingtin with 48 polyglutamine repeats (Fig. 2B).

To determine further whether polyglutamine-expanded huntingtin-induced cell death is apoptotic, an ICE inhibitor (zVAD-fmk) or CPP32 inhibitor (zDEVD-fmk) was added to the medium during transfection (16, 17). Both the ICE and CPP32 inhibitors completely blocked apoptotic cell death induced by expression of mutated huntingtin with 48 or 89 polyglutamine repeats. TUNEL staining was negative (Fig. 2C), and the rate of cell proliferation was similar to the control (transfected with pFL16HD) at both 48 and 72 h after transfection. ICE cleaves inactive CPP32 precursor, thereby activating the enzyme (20). This result therefore suggests that expression of polyglutamine-expanded huntingtin may stimulate ICE, which in turn activates CPP32 to induce apoptotic cell death.

I then explored the possible molecular mechanism of polyglutamine-expanded huntingtin-mediated neuronal toxicity. Since activation of JNK has been implicated in neuronal apoptosis induced by glutamate, kainic acid, or deprivation of neurotrophic factors (9–12), and since glutamate-mediated excitotoxicity may be involved in HD neuronal loss (7, 8), I investigated whether expression of polyglutamine-expanded huntingtin induces activation of JNKS. GST c-Jun (1–89 amino acids) was utilized as a substrate to measure JNK activity. HN33 cells were transfected with pcDNA1, pFL16HD, pFL48HD, or pFL89HD, and 16 h after transfection, cells were lysed, and JNK was precipitated by GST-c-Jun fusion protein beads. A low level of JNK activation was observed in pcDNA1-transfected cells (Fig. 3A, Control). This result is consistent with the data obtained from primary cultures of rat striatal neurons where a basal level of JNK activation was also observed (12). Transfection of normal huntingtin with 16 polyglutamine repeats did not further increase the amount of serine phosphorylated GST-c-Jun and thus did not stimulate JNK activity in HN33 cells (Fig. 3, A and B). Expression of mutated huntingtin with 48 or 89 polyglutamine repeats, however, significantly increased the level of JNK activity. As shown in Fig. 3, A and B, serine phosphorylated GST-c-Jun was increased 7–8-fold 16 h upon transfection of either pFL48HD or pFL89HD, similar to the level of JNK activity induced by 30 min of UV irradiation (Fig. 3, A and B). These results indicate that the polyglutamine repeat expansion of huntingtin enables it to activate JNKS in HN33 cells and the JNK activation precedes apoptotic cell death.

Next, I explored whether activation of JNK is responsible for polyglutamine-expanded huntingtin-induced apoptotic cell death in HN33 cells. JNK is specifically activated by SEK1 (21), and a dominant negative mutant form of SEK1 (K54R) can act as an inhibitor of the wild-type, blocking its phosphorylation and the activation of JNK (22, 23). To investigate the role of JNK activation in polyglutamine-expanded huntingtin-mediated apoptosis in HN33 cells, I examined the effect of co-expression of this dominant negative mutant form of SEK1 with polyglutamine-expanded huntingtin. Transient expres-
sion of either wild-type or dominant negative SEK1 alone had little effect on the proliferation and survival of HN33 cells (data not shown). Co-expression of pcDNA1 with pEBG (SEK1 vector) also did not produce any toxic effect (Fig. 3, C and D). Co-transfection of wild-type SEK1 with pFL48HD or pFL89HD did not affect neuronal toxicity induced by mutated huntingtin (Fig. 3C), while co-expression of dominant negative mutant SEK1, however, significantly prevented apoptotic cell death induced by the mutated huntingtin with either 48 or 89 polyglutamine repeats (Fig. 3, C and D). At 48 h after transfection, about 25–30% of cells had undergone apoptotic cell death compared with ~75% of cells after expression of mutated huntingtin with 48 or 89 polyglutamine repeats alone (Fig. 3, C and D). These TUNEL-negative HN33 cells appeared to be viable, because cells continued to proliferate, and trypan blue staining was negative. I further examined whether the rescuing effect of dominant negative SEK1 is mediated by inhibition of the JNK activation. Co-expression of dominant negative SEK1 significantly attenuated the JNK activity induced by mutated huntingtin with either 48 or 89 polyglutamine repeats (data not shown). These results further support the conclusion that polyglutamine-expanded huntingtin activates the SEK1-JNK pathway to mediate apoptosis in HN33 cells.

**DISCUSSION**

In the present study, I demonstrate that expression of mutated huntingtin containing 48 or 89 polyglutamine repeats activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. The observed neuronal toxicity is unlikely to be due to overexpression of mutated proteins, since expression of the same level of normal huntingtin with 16 polyglutamine repeats does not initiate cell death. Apparently, neuronal apoptosis induced by huntingtin is dependent on expansion of its polyglutamine repeat. Moreover, neuronal toxicity induced by mutated huntingtin with 89 polyglutamine repeats occurs slightly, but consistently, earlier than huntingtin with 48 polyglutamine repeats, further supporting the notion that neuronal apoptosis is mediated by polyglutamine repeat expansion. Since expression of mutated huntingtin alone does not induce apoptosis in 293 cells, this toxic effect may be a cell-specific event.

Selective loss of neurons in different brain regions is a hallmark of HD (2, 4), and neuronal apoptosis is one of the pathological changes observed in brains of HD patients, particularly in the early stage of the disease (24, 25). Thus, understanding of the molecular mechanism of neuronal death in HD is essential for revealing the pathogenesis of HD. My studies first demonstrate that expression of polyglutamine-expanded huntingtin leads to neuronal apoptosis. Since hippocampal neurons are targeted in HD (2, 4), my results are consistent with the major pathological feature of the disease. Moreover, the time
course of neuronal toxicity generated by mutated huntingtin is clearly correlated with the length of the polyglutamine repeat as is the onset of the disease (5, 6).

Activation of JNK appears to be a major factor in the apoptotic death of HN33 cells induced by polyglutamine-expanded huntingtin. Mutated huntingtin with 48 or 89 polyglutamine repeats activates JNK in HN33 cells, while normal huntingtin with 16 repeats fails to do so, suggesting that activation of JNK by huntingtin requires expansion of the polyglutamine repeat. Activation of JNK induced by mutated huntingtin takes place several hours prior to apoptotic cell death, implying that JNK activation may trigger apoptotic pathways. The JNK-c-Jun-mediated apoptosis has been reported to be sensitive to both ICE and CPP32 inhibitors (19, 26), mutated huntingtin-mediated apoptosis was blocked by either inhibitor. Furthermore, dominant negative SEK1 inhibits mutated huntingtin-induced activation of JNKs in HN33 cells and also attenuates apoptotic cell death, further indicating that mutated huntingtin activates the SEK1-JNK pathway to induce neuronal apoptosis in HN33 cells.

Excitotoxicity is thought to contribute a final common pathway of neuronal injury in a wide range of neurodegenerative disorders including HD (7, 27). Administration of NMDA receptor agonists in rats causes selective loss of medium-spiny neurons in striatum that are also particularly affected in HD (8). The most intriguing data comes from gene-targeted knockouts of JNK3, which renders mice resistant to neuronal excitotoxicity mediated by kainic acid receptors (12). Given the fact that activation of kainic acid or glutamate receptors and mutated huntingtin share selective neuronal targets, they could also utilize common cellular mediators for the induction of neuronal toxicity. JNK is one such cellular mediator for neuronal toxicity induced by both mutated huntingtin and glutamate/kainic acid receptors, since my current study and other reports (11, 12) show that activation of JNK is responsible for neuronal toxicity induced either by mutated huntingtin or by stimulation of glutamate/kainic acid receptors.

Currently, it is unclear whether JNK activation precedes neuronal loss in HD patients. JNK activates c-Jun and AP-1 transcription factors to induce apoptosis (9, 27). Increased expression and translocation of c-Jun and NF-κB (often concomitantly activated with the JNK cascade) has been observed in several neurodegenerative diseases (28, 29). Moreover, JNK activation is responsible for neuronal apoptosis induced by a variety of oxidative stress stimuli such as ischemia (30). These data and my current study suggest that overactivation of the JNK cascade may be a common pathway of neuronal death in different neurodegenerative diseases and in acute insults. In summary, my findings show that activation of the SEK1-JNK pathway may mediate neuronal death in HD, and proteins that are involved in activation of this signaling pathway may therefore be potential drug targets for the prevention of neuronal loss in HD as well as other neurodegenerative disorders.

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