A NEW CELLULAR MODEL OF PATHOLOGICAL TDP-43: THE NEUROTOXICITY OF STABLY EXPRESSED CTF25 OF TDP-43 DEPENDS ON THE PROTEASOME


Department of Neurology, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, People’s Republic of China

Institute of Cardiocerebrovascular Disease, West Heping Road 215, Shijiazhuang, Hebei 050000, People’s Republic of China

Neurological Laboratory of Hebei Province, Shijiazhuang, Hebei 050000, People’s Republic of China

Abstract—The C-terminal fragments-25(CTF25) of TDP-43 is a fragment of TAR DNA-binding protein 43 kDa (TDP-43), which is involved in RNA metabolism, neurite outgrowth, and neuronal development and stress granules. Not until recently did evidence suggest that CTF25 might play an important role in amyotrophic lateral sclerosis (ALS) pathogenesis. However, mechanical details on CTF25 causing motor neuron degeneration still remain unknown. To study the toxicity of CTF25 of TDP-43, we established a cellular model stably expressing CTF25 of TDP-43. Herein, we found that stably expressed CTF25 could induce significant oxidative stress and was mainly degraded by the proteasome pathway in cells. Furthermore, the neurotoxicity of CTF25 of TDP-43 was dependent on proteasome activity. In addition, electron microscopy showed mitochondrial swelling and cristae dilation in cells expressing CTF25 and that CTF25 aggregates were characterized by filamentous bundles and electron dense granular material. In conclusion, the new cellular model mimics classical toxic TDP-43 cellular model and interestingly the toxicity of CTF25 is dependent on the proteasome. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: TDP-43, the CTF25 of TDP-43, amyotrophic lateral sclerosis.

INTRODUCTION

TAR DNA-binding protein 43 kDa (TDP-43) is a multifunctional nuclear factor which is involved in many crucial cell processes and neuronal development (Ayala et al., 2008; Bose et al., 2008; Iguchi et al., 2009; Wu et al., 2010; Li et al., 2013). TDP-43-positive inclusions were presented in the degenerative neurons in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitin-positive inclusion (FTLD-U) patients (Neumann et al., 2006). Recent studies have identified that TDP-43 mutations account for 5% of familiar ALS patients and up to 4% of sporadic patients (Pratt et al., 2012). It is still unclear how mutant TDP-43 induced motor neuron degeneration despite much effort. There is a debate among the community on whether gain of toxicity or loss of function plays the major role (Blokhuis et al., 2013). Interestingly, the C-terminal fragments-25 (CTF25) of TDP-43, a fragment of TDP-43, is prone to be misfolded and thus form aggregates (Zhang et al., 2009). It had been reported that overexpression of CTF25 induced mitochondrial autophagy and presented ubiquitin-positive inclusions similar to mutant TDP-43 model (Igaz et al., 2009; Hong et al., 2012). Furthermore, CTF25 of TDP-43 transgenic mice conferred cognitive defects and glucocorticoid, a stress inducer, potentiated the neurotoxicity by increasing levels of CTF25 (Caccamo et al., 2012, 2013).

It has been well recognized that the ubiquitin proteasome pathway (UPP) is responsible for the degradation of biologically so-called “non-useful” proteins, including mutant, misfolded, damaged, terminally modified or over-accumulated proteins (Hershko and Ciechanover, 1998), while autophagy machinery removes surplus, damaged, and often toxic components such as the proteins which aggregate too large to be removed by the proteasome system (Deretic, 2010). Both systems are important for neuron survival and the balance between protein synthesis and degradation. Ubiquitin-positive inclusion is a hallmark for ALS and FTLD-U, indicating that dysfunction of UPP could be involved in the pathogenesis of these diseases. Recent data showed disruption of proteasomes especially in the motor neurons replicated ALS (Tashiro et al., 2012). Activation of autophagy is also one of the most significant features presented in TDP-43-induced neuron degeneration (Chen et al., 2012). Interestingly, autophagy-associated protein P62 was identified in the ubiquitin-positive inclusions, which acted as an autophagy receptor to...
facilitate the degradation of aggregates by the autophagy lysosome pathway (ALP). Two research groups have demonstrated that both UPS and autophagy were involved in the degradation of overexpressed CTF25 of TDP-43 (Urushitani et al., 2010; Wang et al., 2010). However, it is largely unknown whether the stably expressed CTF25 of TDP-43, i.e. consistently expressed CTF25 is toxic and how the two pathways respond to the protein. To gain more valuable information, we established a polyclonal cell line stably expressing CTF25 and found that CTF25 of TDP-43 could induce oxidative stress and was degraded by proteasomes other than autophagy.

EXPERIMENTAL PROCEDURES

Cell lines

NSC-34 is a hybrid cell line that retains the ability to proliferate and express several motor neuron characteristics (Cashman et al., 1992). NSC-34 was routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA; Cat. No: 21063-029) with 10% heat-inactivated FBS (certified performance tested; Invitrogen Corporation, Carlsbad, CA, USA; Cat. No: 16000-044) and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin). Following the manufacturer's protocol, NSC-34 cells were transfected with the empty pCI-neo vector or the vector cloned with the CTF25 of TDP-43cDNAs by using Lipofectamine 2000 transfection reagent (Invitrogen Corporation, CA, USA; Cat. No: 11668-019). NSC-34 cells were routinely maintained at 37 °C in a 5% CO₂ humidified atmosphere in 25 cm² flasks (Corning Incorporated, Corning, NY, USA), changing the medium every 2–3 days.

Chemicals

Z-Leu-Leu-Leu-al, also termed Cbz-LLL or z-LLL (MG132) was purchased from the Beyotime Institute of Biotechnology (Nanjing, China; Cat. No: S1748). Rapamycin was purchased from the Beyotime Institute of Biotechnology (Nanjing, China; Cat. No: S1842). 3-Methyladenine was purchased from Sigma Chemical Company (St. Louis, MO, USA; Cat. No: M9281). G418 was purchased from Sigma Chemical Company (St. Louis, MO, USA; Cat. No: A1720). Lactacystin was purchased from Sigma Chemical Company (St. Louis, MO, USA; Cat. No: A1720). Bafilomycin A1 was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China; Cat. No: 14816).
Fig. 2. The stably expressed CTF25 of TDP-43 induced oxidative stress. (A, B) HO-1 was tested by Western blot in the CTF25 cell line and the control. Average level of relative HO-1 intensity in three independent experiments (mean ± SE) was shown. *P < 0.05. (C) The MDA level was measured in the CTF25 cells and repeated three independent experiments (means ± SE), *P < 0.05.

Fig. 3. CTF25 of TDP-43 expression caused oxidative stress in vitro. ROS was evaluated by MitoTracker® Red CM-H2Xros. Nuclei were stained by Hoechst33342. Empty vector has only the tag of EGFP. The CTF25 of TDP-43 was fused with EGFP. Bar = 20 μm. The intensity was evaluated by Image J software, and at least 30 cells expressing CTF25 were analyzed. *P < 0.05.
Sorting by flow cytometry

The $1 \times 10^8$ NSC-34 cells were harvested after being digested by trypsin (0.25% w/v), and then were transferred to 50 mL conical tubes and centrifuged at 400 g for 5 min. The supernatant was discarded and the pellet was resuspended in cell culture medium. The resuspended cells were aspirated up and down through a pipette several times to help disaggregate clumps. The final cell concentration is adjusted to $10^7$/ml. The purified Green fluorescent protein (GFP)-positive cells were obtained by cell sorting using a FACSAria (BD). The cells that only express GFP were used as the control.

Lipoperoxidation

Levels of lipid peroxidation were tested using the thiobarbituric acid-reactive substances (TBARS) assay kit (Jian Cheng Biological Engineering Institute, Nanjing, China). Lipid peroxidation products, including malondialdehyde and hydroperoxides, could react with thiobarbituric acid and produce a product that can be sensitively measured by a spectrophotometer. Samples were mixed with the reagents at room temperature, then the mixture was incubated at 95 °C for 40 min and cooled down by flow water. Absorbance was read at 532 nm, and TBARS levels were calculated using a standard curve of malondialdehyde bis-dimethyl acetal (MDA). Data are expressed as nmol of TBARS/mg protein. The protein concentration was calculated by the Bradford’s method, using albumin as standard.

Cell viability assay

Cell viability was detected by Cell counting kit-8 (CCK-8) assay. Cells were seeded into 96-well plates at $3 \times 10^3$ cells per well and cultured overnight at 37 °C in a humidified 5% CO2 atmosphere. Then 10 μL of the CCK-8 solution was added to each well of the plate. Cells were incubated at 37 °C for 2 h, and then the absorbance at 450 nm was measured. All experiments
were done in triplicate and repeated eight independent times.

**Western blot**

Cells were rinsed twice with PBS, and then collected by centrifugation at 500g for 5 min at 4 °C. Protein was extracted using a protein extraction kit (Beyotime Institute of Biotechnology, Nanjing, China; Cat. No: #P0013). Protein extracts were quantified using the Bradford method. Twenty micrograms of extracts were denatured at 95 °C for 5 min, loaded onto a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and electrophoresed. After electrophoresis, the proteins in gel were transferred onto a polyvinylidene difluoride membrane (Millipore; Billerica, MA, USA). The membrane was then incubated overnight at 4 °C in the following primary antibodies: rabbit anti-TDP-43 (1:1200; Protein Tech Group, Tucson, AZ, USA; Cat. No: 12892-1-AP), rabbit anti-HO-1 (1:500; Stressgen, Ann Arbor, MI, USA; Cat. No: SPA-895), rabbit anti-p62 (1:1500; Sigma Chemical Company, St. Louis, MO, USA; Cat. No: P0067), rabbit anti-caspase-3 (1:800; Bioworld; Cat. No: BS1518), and mouse anti-β-actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. No: sc-47778). Membranes were then incubated overnight with a fluorescence-conjugated secondary antibody (1:3000). The bands of interest on the membrane were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). The usual green- or red-colored bands were converted to black and white colors for data presentation.

**Fluorescence study**

The degradation of CTF25 was examined in the sorted cells treated with an inhibitor of autophagy or proteasome by inverted fluorescence microscopy. The sorted cells were treated with MG132 (2 μM) or lactacystin (50 μM) for different times, 0, 3, 6, 12, and 24 h, respectively.

To study the morphology of the CTF25 aggregates, the sorted cells were treated with MG132 for 24 h. After the treatment, the cells were washed twice with PBS. The cells were observed by confocal microscopy.

To test whether the CTF25 aggregates colocalized with known aggregation markers or not, the fixed cells were permeabilized by 0.3% Triton X-100 for 30 min. Then the cells were blocked with 10% normal goat serum for 30 min, followed by incubation with primary antibodies (P62, Sigma Chemical Company, St. Louis, MO, USA; Cat. No: P0067, Ubiquitin, Abcam, Cat. No: 7780) at 4 °C overnight and secondary antibodies at room temperature for 1 h. Nuclear counterstaining was stained with Hoechst33342 for 40 min, and finally washed twice with PBS. The cells were observed by confocal microscopy.

Fig. 5. The stably expressed level of CTF25 formed aggregates. The aggregation was tested by laser confocal microscope. The stable cells were treated with MG132 (2 μM) for 24 h. Nuclei were stained by Hoechst33342. EGFP fused with the CTF25 of TDP-43. Bar = 10 μm.
To evaluate the level of reactive oxygen species (ROS), the cells were stained with the prewarmed (37°C) solution containing MitoTracker® Red CM-H2XRos and Hoechst33342, and then incubated for 45 min. After staining, the cells were washed twice with PBS. At the end, the medium was replaced with DMEM without phenol red and the cells were observed by confocal microscopy.

The number of GFP-positive cells and intensity were measured by the Image J software.

**Electron microscopy**

Cells were fixed in 4% glutaraldehyde and treated with 1% OsO4 in 0.1 M PBS. The samples were dehydrated through graded acetones and embedded in EPON 812. Ultrathin sections (70 nm) were made, placed on a copper grid, and stained with uranyl acetate and lead citrate. The samples were observed by transmission electron microscope (TEM, JEM-1230).

**Statistical analyses**

All data are expressed as mean ± SD and analyzed by a one-way ANOVA followed by a Student–Newman–Keuls multiple range test or the Dunn T test. Results were considered significant when \( P < 0.05 \).

**RESULT**

**Stably expressed CTF25 of TDP-43 induced oxidative stress and apoptosis**

In our former study, mutant TDP-43 induced oxidative stress and damaged motor neurons. In addition, TDP-43 mutation formed a lot of fragments, such as TDP-35, CTF25 of TDP-43, etc. Because of the toxicity of CTF25, the cells which overexpressed CTF25 cannot be successfully subcultured. In order to study the toxicity of stably expressed CTF25 on the motor neuron, we established the stable cell lines expressing CTF25 of TDP-43 \[ Fig. 1 \] and the empty pCI-neo vector by the cell sorting technique. The stable cell line can be subcultured and the expression level of CTF25 was almost 47 percent of endogenous TDP-43 \[ Fig. 1B \]. In the stable cell line, the marker of oxidative stress, Heme oxygenase-1 (HO-1) and MDA, increased significantly compared to the normal cells that expressed only Enhanced green fluorescent protein (EGFP) \[ Fig. 2A, B \]. Furthermore, to study the level of ROS, the cells were stained by MitoTracker® Red CM-H2XRos and evaluated by laser confocal microscopy. The level of ROS in cells expressing CTF25 was fivefold higher than the cells expressing the empty vector \[ Fig. 3A, B \]. For the sake of studying the toxicity of stably expressed...
CTF25 of TDP-43 on the motor neuron, cell viability was measured by the CCK-8 assay. Absorbance which was read at 450 nm reflected that cell viability decreased significantly in CTF25 of TDP-43 cells [Fig. 10 C]. Then, the apoptotic marker, caspase-3, was analyzed by Western blot. There was a significant change in CTF25 of TDP-43 cells compared with the empty vector group (\( P < 0.05 \)). The level of caspase-3 was fivefold higher than the empty vector samples [Fig. 10 A, B].

Stably expressed CTF25 of TDP-43 was degraded by the proteasome pathway

It has been proved that the CTF25 of TDP-43 could be degraded by proteasome and autophagy (Caccamo et al., 2009; Wang et al., 2010; Brady et al., 2011; Bose et al., 2011; Wang et al., 2012). To study whether the stably expressed CTF25 was degraded by proteasome, cells expressing CTF25 were treated with MG132 (2 \( \mu \)M) or lactacystin (50 \( \mu \)M) for 3, 6, 12 and 24 h respectively. MG132 is an effective, reversible 26S peptide aldehyde proteasome inhibitor, which can inhibit the proteasome-pathway protein degradation, thereby affecting cell proliferation and promoting apoptosis. Lactacystin is a kind of natural streptomycyes metabolite existing in the soil, and also a selective 20-s inhibitor. Lactacystin and \( \beta \)-lactone of its intermediates selectively and irreversibly bind to the proteasome \( \beta \) subunit, thereby inhibiting the activity of various peptidases. Our results showed that the expression of CTF25 increased gradually and reached the highest level at 12 h. However, no change was observed in the endogenous TDP-43 and GFP of the empty vector [Fig. 4 A, B]. The fluorescence number of CTF25 and GFP of the empty vector was also evaluated by the inverted fluorescence microscopy. We found that the fluorescence of CTF25 increased in a time-dependent manner and the amount of the fluorescence of CTF25 reached the maximum in 24 h [Fig. 4 C, D]. However, no change was observed in the GFP of the empty vector [Fig. 4 C]. Therefore, the stably expressed CTF25 of TDP-43 could mainly be degraded by the proteasome pathway. The CTF25 had a property that it is easy to form aggregation when it was overexpressed. In the stable cell line, we also found that the stably expressed CTF25 formed some small aggregates just like dots near the nuclear membrane. However, after the cells been treated by MG132, the CTF25 formed bigger aggregates located in the cytoplasm and nucleus [Fig. 5]. Moreover, the CTF25 aggregates colocalized with ubiquitin and p62 [Fig. 6]. Furthermore, the aggregates were also evaluated by TEM and were characterized by filamentous bundles and electron dense granular material [Fig. 7 A]. The
The morphology of cells stably transfected with empty vector was also evaluated by TEM. However, no aggregate was detected [Fig. 7B]. It was well known that autophagy played an important role in the degradation of long-lived protein and turned over the aggregates (Cherra et al., 2010; Sarkar et al., 2011; Ułamek-Kozioł et al., 2013). To study whether autophagy was involved in the degradation of stably expressed CTF25, we treated the stable cells by 3-methyladenine (3MA) and bafilomycin A1 which were known as inhibitors of autophagy. 3MA, a widely used autophagy inhibitor, inhibits autophagy by blocking autophagosome formation via inhibition of type III phosphatidylinositol 3 kinases (PI3 kinase). Bafilomycin A1 is a vesicular proton pump inhibitor of enzyme H+-ATP, which is from the gray macrolide antibiotic streptomycin. Bafilomycin A1 can inhibit the formation of late autophagy by inhibiting the formation of vesicles. Meanwhile, the stable cells were also treated with an autophagy activator rapamycin at different doses. In mammalian cells, rapamycin inhibits the kinase activity of mammalian target of rapamycin (mTOR) by forming a complex with the immunophilin FK506-binding protein of 12 kDa (FKBP12) that binds to and inactivates mTOR, leading to the upregulation of autophagy. Surprisingly, the expression of CTF25 was neither affected by autophagy inhibitor 3MA, bafilomycin A1 nor autophagy activator rapamycin [Fig. 8]. There was no significant change in EGFP and CTF25 expression in all the study groups. Therefore, the stably expressed CTF25 of TDP-43 was mainly not degraded by the autophagy pathway.

The toxicity of the CTF25 of TDP-43 depends on the proteasome activity

Since CTF25 expression was not affected by the autophagy pathway, we further examined a proteasome inhibitor, MG132. Interestingly, MG132 increased the expression of CTF25 of TDP43, indicating their degradation via the proteasome pathway. Moreover, the expression of HO-1, an inducible enzyme in response to stress, was significantly increased. Mitochondria has been regarded as a place where ROS was produced. Next, we investigated the mitochondrial changes in the CTF25 expressing cellular model by TEM. Our results
showed mitochondrial swelling and cristae dilation in the CTF25-expressing stable cells. Furthermore, the mitochondria became more swollen after application of the proteasome inhibitor MG132 [Fig. 9].

DISCUSSION

In this paper, we established a new cellular model stably expressing CTF25 of TDP-43. We found that the stable cell line demonstrated some features of pathological TDP-43, including ubiquitinated aggregates, oxidative stress, and mitochondria dysfunction. Firstly, the stably expressed CTF25 was mainly degraded by the proteasome pathway, not autophagy pathway, even if it formed aggregates. It is well-known that the protein which is prone to form aggregates is degraded by both UPP and ALP (Urushitani et al., 2010; Wang et al., 2010). But, we found that the proteasome pathway preferred to degrade CTF25 in the stable cell line. This finding might suggest that the proteasome could play an essential role in the aggregates forming, especially at the early phase. TDP-43 or C-terminal fragments of TDP-43 were easy to form the aggregates when the function of the proteasome was inhibited, especially in the presence of TARDBP mutations. Recently, other papers reported that conditional knockout mice of the proteasome subunit Rpt3 in a motor neuron-specific manner showed motor neuron death and formed aggregates positive for TDP-43, FUS (fused in sarcoma), ubiquitin 2, and optineurin (Tashiro et al., 2012). Notably, autophagy is responsible for the degradation of

Fig. 9. The toxicity of CTF25 of TDP-43 is regulated by proteasome. (A, B) HO-1 was tested by Western blot in the CTF25 cell line and the control. Average level of relative HO-1 intensity in three independent experiments (mean ± SE) was shown. *P < 0.05. The mitochondrial morphology was evaluated by the TEM. Scale bar = 500 nm.
aggregates (Urushitani et al., 2010; Cecarini et al., 2014). However, we did not find that autophagy was involved in the degradation of CTF25. So, whether UPP or ALP is involved in the process of aggregates degrading may depend on the state of the CTF25: if stably expressed, UPP is set to a preferred position.

Secondly, we found the stably expressed CTF25 of TDP-43 induced oxidative stress and damaged the mitochondria. Oxidative stress and the dysfunction of mitochondria have been studied in the pathological TDP-43-related disease, such as ALS, FTLD and Alzheimer's disease, which are common features in the process of neuron degeneration (Mizushima et al., 2008; García et al., 2013). We had described mutant TDP-43-induced oxidative stress participates in motor neuron degeneration (Duan et al., 2010). But, the mechanism is not clear. Recent papers showed that mutant TDP-43 is a resource of fragments of TDP-43, such as CTF25 of TDP-43 (Sreedharan et al., 2008). In addition, CTF25 induced mitochondrial autophagy and ROS in vitro and resulted in forelimb impairment in vivo (Hong et al., 2012; Dayton et al., 2013). Thus, the neurotoxicity induced by the CTF25 might be one of the most important parts of pathogenesis of mutant TDP-43. The dysfunction of proteasome in the neurons resulted in the protein aggregates and cellular apoptosis (Jara et al., 2013). The stably expressed CTF25 is mainly degraded by the proteasome. So, the proteasome might be a potential target to reduce the toxicity of the stably expressed CTF25 of TDP-43.

A major supplement in our understanding of ALS pathogenesis started in 2006 with the identification of the TDP-43 as the main component of ubiquitinated protein aggregates found in sporadic ALS patients and in patients with FTLD. Immunoblotting of protein extracted from the affected brain and spinal cord has defined a biochemical signature of disease that includes the production of several CTFs around 25 kDa (Arai et al., 2006). In cells of N2A and SH-SYSY, the overexpression of the CTF25 is sufficient to induce the cytoplasmic accumulation of TDP-43 (Caccamo et al., 2009). In transgenic mice expressing wild-type or mutant TDP-43, the appearance of CTF25 was shown to accelerate the disease progression, suggesting that CTF25 of TDP-43 may be involved in the disease pathogenesis (Wils et al., 2010).

In summary, our experiments show that the new cellular model mimics some features of pathological TDP-43 and the toxicity of the stably expressed CTF25 of TDP-43 is dependent on the proteasome. The cellular model described above should be useful for studying the pathogenesis of ALS and for evaluating novel treatments.

REFERENCES


