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Dynamic expression of Hsp27 in the presence of mutant ataxin-3

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Abstract

Machado–Joseph disease (MJD)/spinocerebellar ataxia type 3 (SCA3) is an autosomal dominant spinocerebellar degeneration characterized by a wide range of clinical manifestations. The molecular mechanisms underlying the selective neuronal death typical of MJD/SCA3 are unknown. In this study, human SK-N-SH neuroblastoma cells stably transfected with full-length MJD with 78 CAG repeats were assayed for the dynamic expression of Hsp27, known as a suppressor of poly-Q mediated cell death, in the presence of mutant ataxin-3 in different passages of cultured cells. A dramatic decrease of Hsp27 expression was observed in the earlier passage of cultured SK-N-SH-MJD78 cells, however, the later passage of cells showed a significant increase of Hsp27 to almost the same level of the parental cells. Furthermore, immunohistochemical analysis of MJD transgenic mice and post-mortem human brain tissues showed increased expression of Hsp27 compared to normal control brain, suggesting an up-regulation of Hsp27 in the end stage of MJD. However, mutant cells of earlier passages were more susceptible to serum deprivation than mutant cells of later passages, indicating weak tolerance toward stress in cells with reduced Hsp27. While heat shock was used to assess the stress response, cells expressing mutant ataxin-3 displayed normal response upon heat shock stimuli when compared to the parental cells. Taken together, we proposed that during the early disease stage, the reduction of Hsp27 synthesis mitigated the ability of neuron cells to cope with cytotoxicity induced by mutant ataxin-3, triggering the cell death process during the disease progress. In the late stage of disease, after prolonged stressful conditions of polyglutamine cytotoxicity, the increased level of Hsp27 may reflect a dynamic process of the survived cells to unfold and remove mutant ataxin-3. However, this increased Hsp27 still cannot reverse the global dysfunction of cellular proteins due to accumulation of cytotoxic effects.

Keywords: Spinocerebellar ataxia type 3; Full-length mutant ataxin-3; Heat shock protein 27; Heat shock response

Spinocerebellar ataxia type 3 (SCA3)/Machado–Joseph disease (MJD), one of the dominantly inherited neurological diseases [1], is related to a common pathological mechanism with a polyglutamine expansion within the relevant disease protein. Polyglutamine-related diseases are dominantly inherited, typically late-onset, and fatal neurodegenerative disorders. Patients usually develop a slowly progressive movement disorder and die within 10–20 years after onset. These diseases affect various central nervous system (CNS) structures, but all of them eventually lead to brain stem dysfunction. Clinically, SCA 3 is characterized by a progressive ataxia in combination with various non-cerebellar symptoms, including oculomotor abnormalities, spasticity, basal ganglia symptoms, peripheral neuropathy, and cognitive disturbances [2,3]. All affected

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SCA3 patients exhibit expanded CAGs with 55–84 repeats whereas normal individuals exhibit 13–51 repeats [4]. The protein, ataxin-3, is widely expressed in neurons and outside the CNS and mutations ultimately lead to a selective neuronal loss in restricted brain regions [5]. The nature of the toxic insult of a poly-Q mutation and its biological consequences for the disease are unclear. Several studies have demonstrated that protein fragments containing an expanded polyglutamine possess an increased vulnerability to apoptotic death. It is possible that the poly-Q expansion interferes with basic cellular processes such as transcription, protein degradation, and survival/death signaling [6]. Genetic and molecular studies have suggested that poly-Q causes altered gene expression, abnormal protein interactions, alteration of proteolysis, and activation of caspases and protein unfolding [7–11]. However, the causal relation between these cellular events and the pathogenesis has not been elucidated.

Expanded polyglutamine aggregates, both in vitro and in vivo, form characteristic inclusion bodies. It was shown that the ataxin-3 accumulated in ubiquitinated intranuclear inclusions selectively in neurons of affected brain regions [12]. Neuronal intranuclear inclusions have become the neuropathological sign at the late stage of polyglutamine diseases, but the relationship between aggregation and cytotoxicity is currently unclear [13,14]. There is a possibility that the expanded polyglutamine tract may destabilize the protein to misfold and aggregate. Therefore, protein chaperones, also known as heat shock proteins (HSPs) which help restore proteins to their native conformation after they have been misfolded due to heat, ischemia, chemotoxicity or other cellular stressors [15], have drawn researchers' attention in recent years [6,16,17]. HSPs or stress proteins participate in protein synthesis, protein folding, transport, and translocalization processes. Stress situations trigger a heat shock response leading to their induction. The up-regulation of stress proteins is an important step in prevention of protein aggregation and misfolding after stress, and is also essential during development and differentiation [18]. Hsp70 and Hsp40 can function in an ATP-dependent process to catalyze the refolding of denatured or partially denatured modules into enzymatically active forms [19]. In SCA3 brain, Hsp40 and Hsp70 were found to localize to nuclear inclusions (NIs) [20,21]. In addition, it was demonstrated that over-expressed chaperones in cells and in fruitfly also reduce the size of the aggregates and even suppress polyglutamine toxicity [20,22–24]. It is worth noting that NIs, as a hallmark of neurodegeneration, were mostly found at the late stage of disease or in postmortem patients' brain tissue.

Hsp27 is a powerful ATP-independent chaperone in vitro, which inhibits aggregation and promotes the refolding of denatured proteins [25]. Hsp27 is expressed in various cell types and tissues, at specific stages of differentiation and development [18], and the failure to obtain knockout mice suggests that this protein is essential for development. The expression of Hsp27 was shown to enhance the survival of mammalian cells exposed to a number of cytotoxic agents, including heat shock, oxidative stress, staurosporine, ligation of the Fas/Apo-1/CD95 death receptor, chemotherapeutic agents, and cytokines [26–30]. Paradoxically, such stimuli often induce Hsp27 over-expression, providing an example of how pro-apoptotic stimuli can elicit protective responses when delivered below a threshold level. Hsp27 was reported to protect cells against oxidative stress and have anti-apoptotic properties in neuronal survival [31–33], which may be partly due to a decrease in reactive oxygen species (ROS) and an increase in glutathione (GSH) [32,34]. In addition, oxidative stress induced by ROS or free radicals played an important role in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease, Huntington disease (HD), and other late-onset neurodegenerative conditions [35,36]. It was reported that over-expression of Hsp27 prevented cellular polyglutamine toxicity and suppressed the increased levels of cellular ROS caused by huntingtin [37]. It is likely that expression of Hsp27 suppresses the cell death induced by expression of expanded polyglutamine via downregulation of the oxidative stress pathway. In our laboratory, we have established stably transfected neuronal cells expressing full-length mutant ataxin-3 [11]. It is interesting to note the ability of neuronal cells to withstand the existence of expanded ataxin-3 for prolonged periods without apparent adverse effects in our cellular model. With very few SK-N-SH-MJD78 cells containing nuclear aggregates, our model system represents an earlier stage of the disease, which may be particularly important for studying the initiation events of polyglutamine cytotoxicity in human neuronal cells. However, the accumulation of the toxic polyglutamine protein might have compromised viability. We previously showed that the protein levels of Hsp27 decreased dramatically in the presence of mutant ataxin-3 when compared to cells without mutant ataxin-3 in neuronal and non-neuronal cells [11]. It is reasonable to speculate that during the early disease stage, the reduction of Hsp27 synthesis mitigated the ability of cells to cope with cytotoxicity induced by the presence of mutant ataxin-3. However, Schmidt et al. [21] observed no Hsp27-positive NIs in pontine sections of SCA3 patients while another study of Evert et al. [38] demonstrated that 17% of the ataxin-3-positive NIs colocalized to Hsp27-positive nuclear aggregates in pontine neurons of SCA3 patients. Therefore, we speculated that even though the reduction of Hsp27 represented an early event in the disease cellular model [11], the expression of Hsp27 might be triggered after the prolonged exposure of the mutant ataxin-3. To confirm this hypothesis, in this study we investigated the expression of Hsp27 not only in different passages of the cells expressing mutant ataxin-3 but also in transgenic mouse and patient' postmortem brain tissues. In addition, expression of Hsps under heat shock stress was evaluated in neuroblastoma cells with and without mutant ataxin-3.

Materials and methods

Materials. SK-N-SH cells were provided by Dr. Shin-Lan Hsu (Taichung Veterans General Hospital, Taiwan). Cell line SK-N-SH-MJD78 was established previously [11]. All culturing supplies and transfection materials were obtained from Gibcol Life Technologies (Gaithersburg, MD, USA). Brain sections from MJD transgenic and non-transgenic mice were prepared previously [39]. Paraffin-embedded brain tissue from one patient with molecular confirmation of a SCA3 mutation and human brain tissue from age-matched normal donors were employed in this study. Western blot reagents were obtained from Pierce (Rockford, USA). Mouse monoclonal anti-MJD was made previously [40]. Polyclonal anti-Hsp27 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-β-actin was from Sigma (St. Louis, MO, USA). Mouse monoclonal anti-hemagglutinin epitope (HA) was from Roche Molecular Biochemicals (Mannheim, Germany). Rabbit polyclonal anti-hemagglutinin epitope (HA) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other supplies were obtained from Sigma Chemical (St. Louis, MO, USA).

Immunocytochemical staining. For immunocytochemical staining, SK-N-SH or SK-N-SH-MJD78 cells were fixed in 4% paraformaldehyde for 20 min, following by three washes in phosphate-buffered saline (PBS), 20 min incubation with 1% Triton X-100 in PBS to permeabilize the cells, 20 min incubation with 2% bovine serum albumin in PBS to block non-specific binding, and then incubation with each antibody at 4 °C overnight. After washing with phosphate-buffered saline, immunodetection was performed with biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin (BioGenex). Finally, the reactants were developed with 3,3′-diaminobenzidine (DAKO). Counterstaining was performed using hematoxylin. Visible aggregate formation was determined using a Ziesse fluorescence microscope equipped with a digital camera. The percentage of cells exhibiting aggregates was calculated. In each experiment, at least 30 cells were counted per dish, with a minimum of four dishes utilized for each time point.

Immunohistochemical staining. After removing wax with xylene and rehydration with 100, 75, and 50% water, the sections were treated 3% H_2O_2 and hot 1/1000 NP-40. Tissue sections were blocked with 5% rabbit serum/PBS for 1 h. Then, they were incubated with polyclonal Hsp27 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After primary antibody incubation, sections were washed and incubated for 1 h at room temperature with the appropriate biotinylated anti-goat IgG. Finally, the sections were treated streptavidin-HRP and Substrate-Chromogen Solution (DAKO LSAB2 System) for staining. The stained sections were counterstained with Mayer's hematoxylin (MERCK).

Lactate dehydrogenase release assay. Lactate dehydrogenase (LDH) released into the medium was assayed by using a LDH Cytotoxicity Detection Kit (Takara Bio Inc.). Different passages of mutant cells and the parental wild type cells were seeded at 5×10^3 cells/well in 96-well plates and allowed to attach for overnight. After cells attached, medium was changed to serum free- and phenol red-free medium. The activity of LDH present in the culture medium was then evaluated at intervals of every 12 h, according to the manufacturer's instructions. Briefly, 50 μl of culture supernatant was transferred to enzymatic assay plate. Then, Substrate Mix was added to each of the plates and incubated at room temperature for 30 min. After the addition of stop solution, the absorption values at 490 nm were determined with an automatic microtiter plate reader.

Heat shock response. SK-N-SH and SK-N-SH-MJD78 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and were then prepared at a concentration of 1.5×10^5 cells/ml, and 2 ml of which was added to each well of 6-well plates. One day after seeding, cells were heat shocked at 42 °C for 1 h. Appropriate controls were included without heat shock treatment. Cells were harvested 0, 5, 10, 15, and 20 h after heat shock and then Western blot analysis was performed.

Western blot analysis of protein levels. In brief, cell lysates containing 20–30 μg protein were loaded onto 12% SDS–polyacrylamide gels. Resolved proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking the membrane with 5% non-fat milk in NaCl/Pi/0.1% Tween 20 for 1 h at room temperature, all antibody-binding reactions were performed in the same buffer supplemented with 1% non-fat milk at 4 °C overnight for primary antibodies and at room temperature for 1 h for secondary antibodies coupled to horseradish peroxidase. The signal was detected by the enhanced chemiluminescence Supersignal System (Pierce, Rockford, USA). Monoclonal antibody against β-actin was included in the experiments as an internal control.

Statistical analysis. All bands were quantified by laser densitometry. All values were expressed as means ± SEM. Analysis of variance with subsequent Student's t test was employed to determine the significance of differences in comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Expression of mutant ataxin-3 in different passages of human neuroblastoma SK-N-SH-MJD78 cells

Human SK-N-SH cells, containing endogenous normal ataxin-3 with 26 glutamine residues, were used as the parental cells. SK-N-SH cells stably transfected with full-length poly-Q expanded MJD construct, SK-N-SH-MJD78, were established from the previous study [11]. Expression of the exogenous mutant ataxin-3 in different passages of SK-N-SH-MJD78 was confirmed by Western blot analysis. A representative Western blot is shown in Fig. 1. When immunoblotted by monoclonal antibody to ataxin-3, the results showed that both normal and expanded (26 and 78 residues) ataxin-3 could be stably expressed in different passages of the transfected cells (Fig. 1). The expanded ataxin-3 was also probed with 1C2 (data not shown). Our results demonstrated that the size and stability of the exogenously expressed ataxin-3 obtained from cells at the 32nd, 68th, and 106th passages remained unchanged. The intensity of the immunoreactive band in cells bearing the mutant ataxin-3 did not significantly alter with time in vitro, up to over the 140th passage (data not shown). In addition, the size of the immunoreactive band remained the same, indicating that the poly-Q repeat was stably replicated in SK-N-SH-MJD78 cells. To date, SK-N-SH-MJD78 cells have been passaged for over 170 passages under normal growth conditions without apparent loss of phenotypes. The transfected cells with different passages (earlier or later generations) were used for the following experiments.

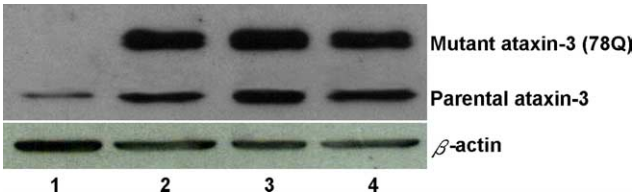

Fig. 1. Detection of expressed wild type and expanded ataxin-3 in human neuroblastoma SK-N-SH-MJD78 cells. Western blot analysis of parental SK-N-SH (lane 1), the stably transfected cells of passages 32 (lane 2), 68 (lane 3) and 106 (lane 4) was performed. Monoclonal anti-ataxin-3 was used in the blotting.

At the same time, immunocytochemical analysis was performed to examine the subcellular localization of the mutant ataxin-3. By probing with anti-HA antibodies, the results from immunocytochemical study showed that full-length ataxin-3 with expanded repeat (78 residues) usually diffusely distributed in the cytoplasm of the 88th passage of transfected cells (Figs. 2C and D), compared with very little background staining in the control cells (Figs. 2A and B). It was noted that mutant ataxin-3 accumulated in the cytoplasm or the perinuclear (Figs. 2E and F) location in the cells of passage 120th. Our results demonstrated that the continuous culture of MJD neuroblastoma cell line to over 100 passages presents a cellular environment with aggregation of ataxin-3 (Figs. 2E and F), although very few nuclear inclusions (NIs) were observed in later passages of mutant cells, which is consistent with what we observed previously [11]. These data indicated that immortalized cells with mutant ataxin-3 expression could be used as a cellular model to study the long-term exposure of the cytotoxic effects accompanied by full-length mutant ataxin-3.

Expression of Hsp27 in cells expressing full-length mutant ataxin-3

Due to the late-onset feature of the disease, we hypothesized that the expression of Hsp27 might be fluctuated during the disease progression. To test this hypothesis, we next investigated whether the expression of Hsp27 in different passages of mutant cells exhibited different levels of protein expression. Western blot analysis was performed

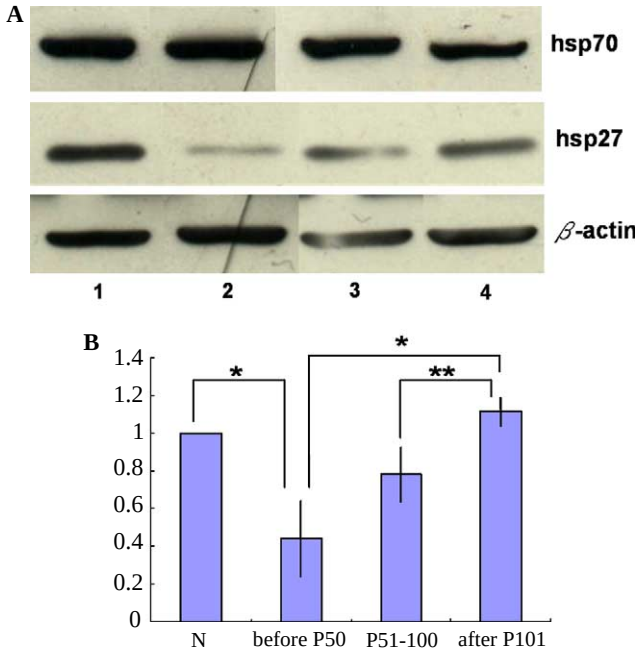

Fig. 3. Hsp27 expression in the cells stably transfected with expanded ataxin-3 or the parental cells. (A) Western blot analysis of Hsp27 proteins. Untransfected SK-N-SH cells (lane 1), the 48th passage of stably transfected SK-N-SH-MJD78 cells (lane 2), the 91st passage of SK-N-SH-MJD78 cells (lane 3), and the 143rd passage of SK-N-SH-MJD78 cells (lane 4) were cultured in complete medium. Cell lysates were electrophoresed, blotted, and probed with anti-Hsp27 antibody and anti-β-actin antibody. (B) Hsp27 expression in the stably transfected cells relative to wild type control. Data from at least three independent experiments with standard errors are shown. N, normal wild type control; before P50, cells before passage 50; P51-100, cells between passage 51 and 100; after P101, cells after passage 101. $^{*}p < 0.002$, $^{**}p < 0.02$.

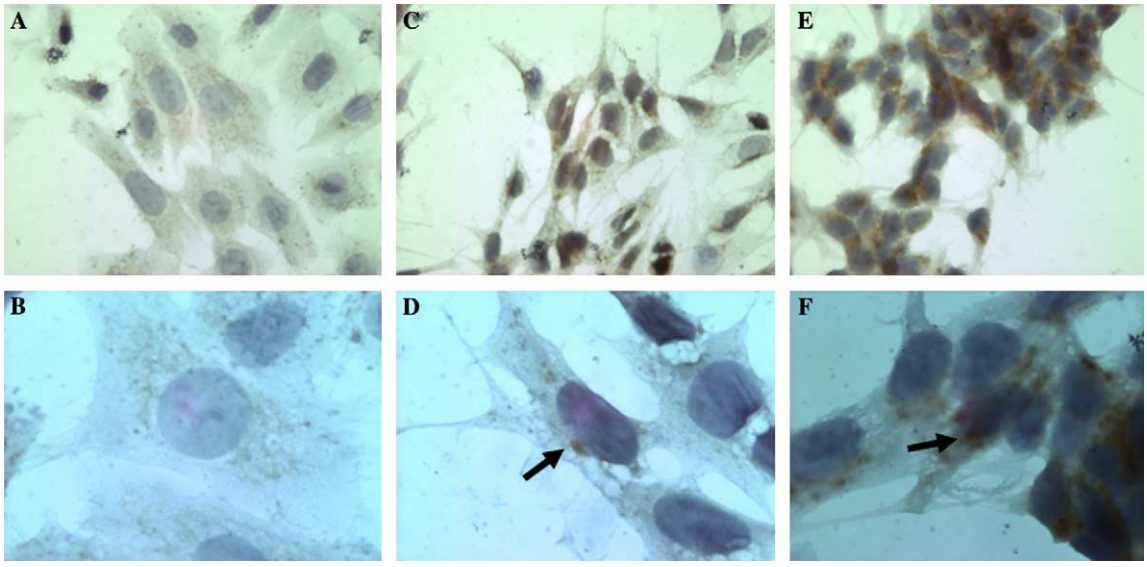

Fig. 2. Immunocytochemical analysis of expressed expanded ataxin-3. (A) SK-N-SH (magnification: 400×). (B) SK-N-SH (magnification: 1000×). (C) The 88th passage of SK-N-SH-MJD78 cells (magnification: 400×). (D) The 88th passage of SK-N-SH-MJD78 cells (magnification: 1000×). (E) The 120th passage of SK-N-SH-MJD78 cells (magnification: 400×). (F) The 120th passage of SK-N-SH-MJD78 cells (magnification: 1000×). Cells were labeled with monoclonal anti-HA antibody.

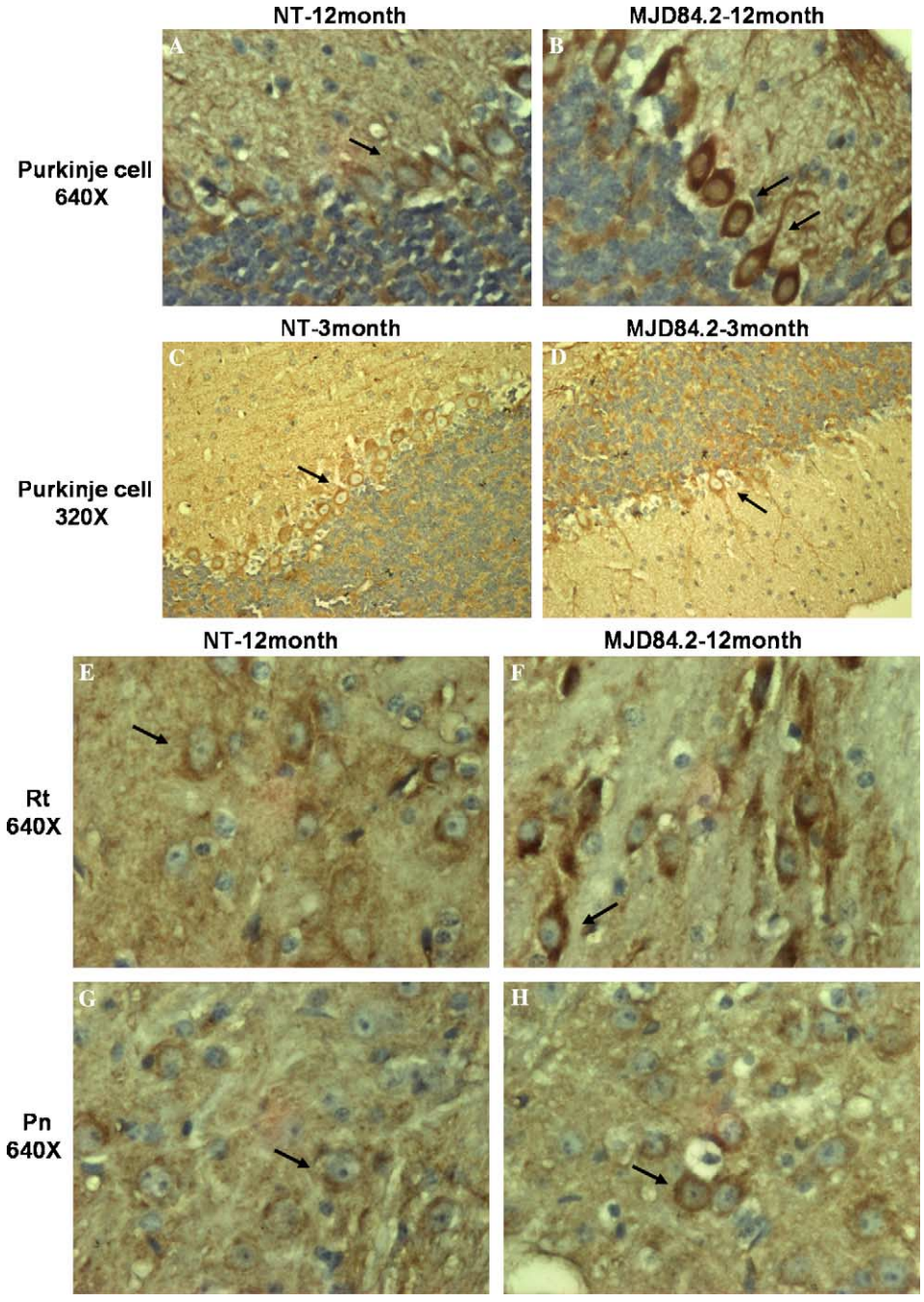

Fig. 4. Hsp27 immunostaining of comparative sections of non-transgenic mouse and MJD84.2 mouse at 3 months or 12 months of age. Microscopic sections were cut at 7 μm and stained with Hsp27 and hematoxylin. Rt, reticular thal nu; Mo5, motor trigeminal nu; Pn, pontine nuclei; DG, dentate gyrus.

in cell extracts from different passages of stably transfected cells by polyclonal antibody against Hsp27. A representative Western blot is shown in Fig. 3A. The results demonstrated that the protein levels of Hsp27 in the 48th passage of stably transfected cells (lane 2 of Fig. 3A) significantly decreased compared with those of parental cells (lane 1 of Fig. 3A). However, the Hsp27 levels of the 91st and the 143rd passages (lanes 3 and 4 of Fig. 3A) increased accordingly. Our results indicated that the expression of Hsp27 may be triggered by the long-term exposure of stress situations caused by mutant ataxin-3. However, the expression of Hsp70 was intact among these different passages of mutant cells (Fig. 3A). A quantitative assessment of the percentage of protein expression revealed that Hsp27 in the stably transfected cells before passage 50 retained only about 40% of that in the parental cells. However, the expression levels of Hsp27 beyond passage 100 restored to almost the same level as the wild type control

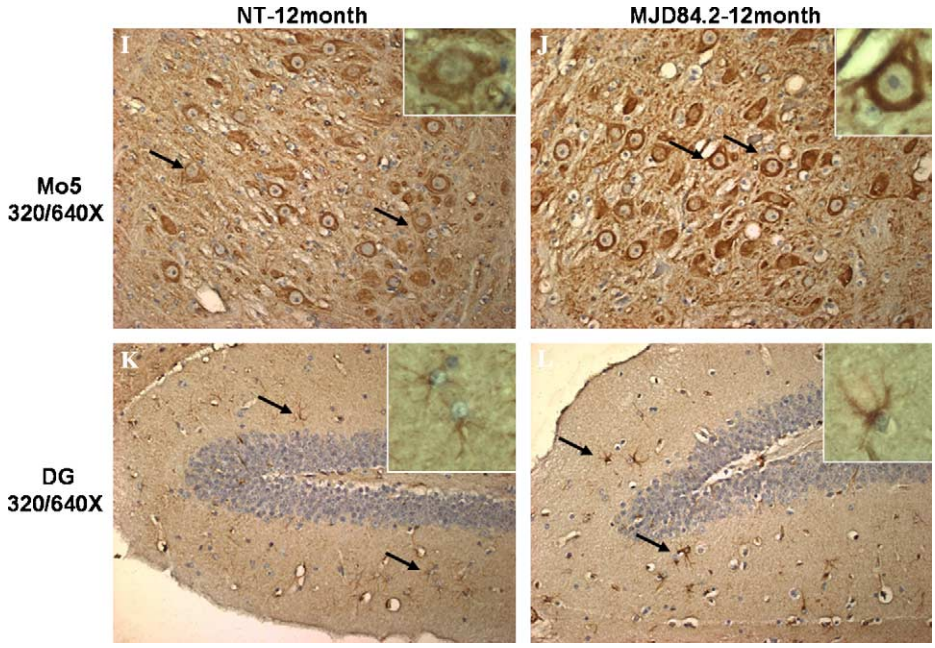

Fig. 4. (*continued*).

(Fig. 3B). This statistical analysis further confirmed a dynamic expression of Hsp27 in the presence of mutant ataxin-3 in the in vitro cellular system.

Expression of Hsp27 in MJD transgenic mice and postmortem MJD brain tissues

In order to investigate whether the expression of Hsp27 was significantly induced in the end stage of disease, brain tissue sections from MJD transgenic mice [39] and a MJD patient were used for immunohistochemical analysis. Immunohistochemical staining was performed using polyclonal Hsp27 antibodies in non-transgenic and MJD84.2 mice [39], which carry two copies of the transgenes with 84 CAG repeats in human MJD genes, as illustrated in Fig. 4. The more intense Hsp27 staining of a subset of cells in the 12-month-old transgenic mice tissues was observed in Purkinje cells, Rt, and Mo5, as shown in Figs. 4B, F, and J, compared to a less intense staining in those of the age-matched non-transgenic control (Figs. 4A, E, and I). It was estimated that about 15% of the neuron cells on the pontine tissue section revealed an increased cytoplasmic expression of Hsp27 (Fig. 4H) compared to about 4.7% of the respective control section (Fig. 4G). In addition, increased numbers of reactive astrocytes were detected with Hsp27 immunostaining in the DG of transgenic mice (Figs. 4K and L). Interestingly, it is noted that almost equal intense staining was observed in Purkinje cells of non-transgenic and MJD84.2 mice of 3 months of age (Figs. 4C and D). The results are consistent with what we speculated that the expression of Hsp27 in the earlier stage of disease is less than that of the later stage. Meanwhile, immunostaining of post-mortem SCA3 brain (cerebellar) tissues was also performed to further confirm this observation. In agreement with what we observed in the 12-month-old MJD transgenic mice brain sections, Hsp27 immunostaining revealed an elevated increased cytoplasmic immunoreactivity in the cerebellum white matter (Fig. 5B) and a mild increase in Purkinje cells of human SCA3 brain (Figs. 5D and F), compared to a less intense staining pattern in the age-matched control brain (Figs. 5A, C, and E).

Mutant ataxin-3 cells from early and late passages differentially affect cell viability upon serum deprivation

Based on the aforementioned fact, it is likely that continuous passages of mutant ataxin-3 cells resulted in growth selection for survived cells with elevated expression of Hsp27. The up-regulation of Hsp27 may be a cell defense mechanism in an attempt to prevent protein aggregation and misfolding during the disease progression. To understand the cell viability of mutant cells of different passages, serum deprivation assays were performed. Different passages of mutant cells as well as the parental wild type cells were cultured without serum. LDH release assay was performed to analyze the cell death at the different intervals. As shown in Fig. 6, the cell viability of the parental and different passages of mutant cells gradually diverged after culturing for 72 h. The parental wild type cells showed higher tolerance for serum deprivation than the mutant cells. In addition, the earlier passage of mutant cells (the 53rd passage) was more susceptible to serum deprivation, compared to the later passage of mutant cells (the 148th passage). These results demonstrated that the altered expression of Hsp27 may have an important modulatory role in the viability of disease cells under stressful condition.

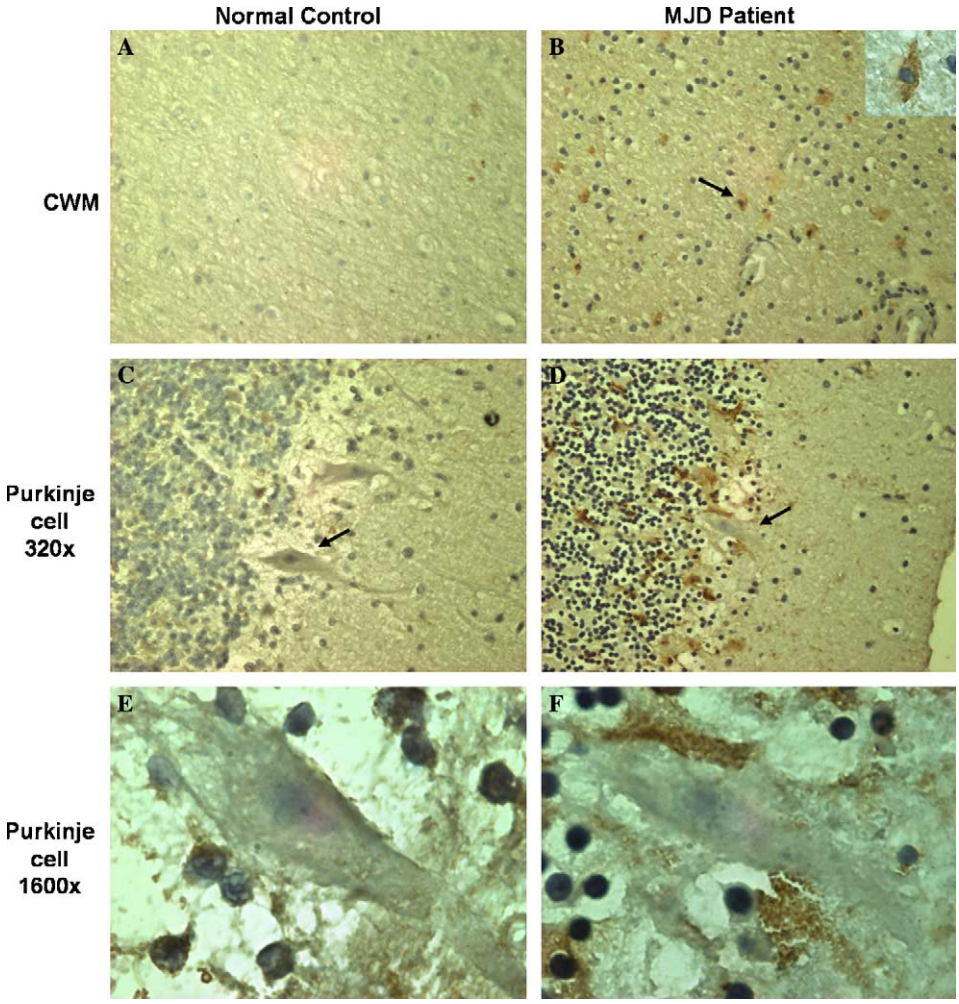

Fig. 5. Immunohistochemical analyses of differentially expressed Hsp27 in human SCA3 disease and control brain tissue. Hsp27 immunostaining of control cerebellar white matter (CWM) (A) compared to disease CWM (B and inset) showed several neurons with increased cytoplasmic immunoreactivity (B and inset). Hsp27 immunostaining of control purkinje cells (C,E) compared to disease Purkinje cells (D,F) showed mild increased cytoplasmic immunoreactivity of Hsp27 in the disease sample (D,F).

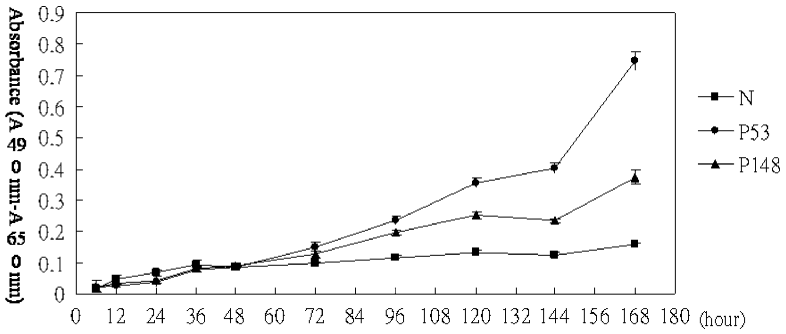

Fig. 6. LDH release assay under serum deprivation. Parental SK-N-SH cells (■), the 53rd passage of SK-N-SH-MJD78 cells (●), and the 148th passage of the mutant cells (▲) were cultured under serum free medium and the release of LDH into culture medium was detected every 12 h. Data represent the average and standard error of three experiments.

Heat shock response is normal in cells expressing full-length mutant ataxin-3

It is possible that the prolonged exposure of mutant ataxin-3 gradually triggers a heat shock response leading to the induction of Hsp27. But why do cells with mutant ataxin-3 eventually die? Is it possible that the presence of mutant ataxin-3 during the cell culturing process mitigates the heat shock response and thus deteriorates the cellular response to the stresses? To assess the functional

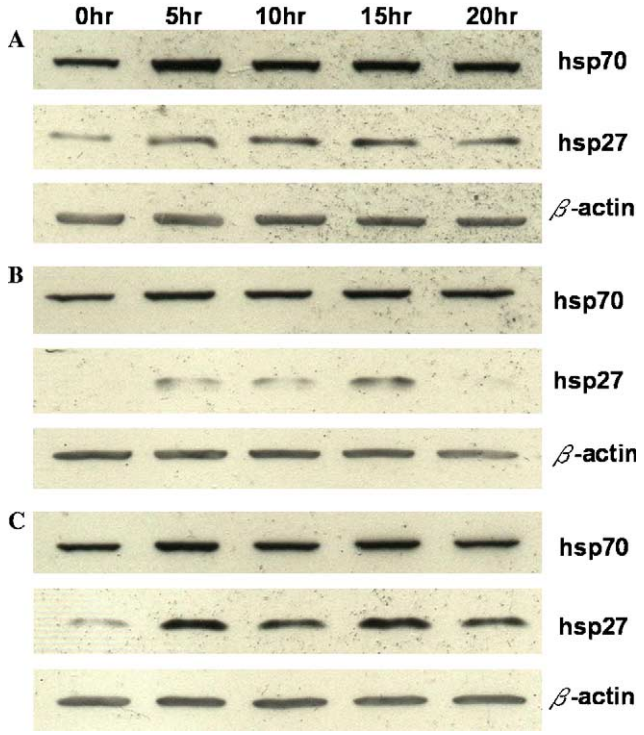

Fig. 7. Western blot analysis of Hsp27 and Hsp70 in the cells with or without stably transfected expanded ataxin-3 under heat shock treatment. Expression of Hsp27 and Hsp70 was studied by immunoblot in whole-cell lysates from control cells (A), the 67th passage of SK-N-SH-MJD78 cells (B) or the 127th passage of SK-N-SH-MJD78 cells (C), either left untreated or heat-shocked 1 h at 42 °C, then incubated 0, 5,10, 15, and 20 h at 37 °C for recovery. Following SDS–PAGE and Western blotting, Hsps were detected immunologically by probing with appropriate specific antibodies.

significance of the Hsp27 under the heat shock stress, we then carried out a time-course analysis of Hsp27 expression in extracts from heat-shocked cells. In brief, the wild type SK-N-SH and different passages of SK-N-SH-MJD78 cells were heat shocked for 1 h at 42 °C, and then cells were harvested at 0, 5, 10, 15, and 20 h after recovery at 37 °C. As shown in Fig. 7, the expression of Hsp27 showed a significant increase at 5 h after heat shock, remained at the plateau until 15 h after heat shock, and then showed a decrease at 20 h after heat shock. It is noted that in both the earlier or later passages of mutant cells, the trend of heat shock response is similar to that in cells containing the normal ataxin-3. Our results demonstrated that even though the expression of Hsp27 was dynamic during the cell culturing process, the cells expressing mutant ataxin-3 in different passages still showed normal response to heat shock stress.

Discussion

As we reported previously [11], the reduction of Hsp27 was observed in the early disease stage of the MJD neuroblastoma cellular model. The same reduction of Hsp27 expression was also observed in COS-7 cells and lymphoblastoid cells stably expressing mutant atxin-3 compared to that in cells stably expressing non-mutant ataxin-3 ([11] and data not shown). In addition, SK-N-SH cells expressing mutant ataxin-3 are more susceptible to exogenous oxidative stress, evidenced by a higher incidence of death when submitted to oxidative stress [11]. Because Hsp27 was reported to protect cells against oxidative stress and have anti-apoptotic properties in neuronal survival [31–33], it is possible that a reduction of Hsp27 in the early disease stage plays an important role in triggering the disease onset. As shown in Fig. 6, the 53rd passage of mutant cells exhibits a higher death rate upon serum deprivation, compared to that of the 148th passage. It is very likely that in vivo the neuron cells with mutant ataxin-3 are more susceptible to polyglutamine cytotoxicity, at least partly, because of the reduction in Hsp27 expression. Therefore, the majority of neuron cells of affected patients die at the early stage of disease. Although we do not know yet about the detailed mechanism leading to the reduction of Hsp27 in the early stage of disease, our preliminary results indicated that the defects may be at the translational level (data not shown).

In the present study, we showed that the expression of Hsp27 in the cellular model is gradually increased during the continuously culturing process (Fig. 3). Consistently, the results from immunohistochemical staining of MJD transgenic mice and post-mortem MJD brain (Figs. 4 and 5) further confirmed that in the end stage of the disease, the expression of Hsp27 is more pronounced than in the controls. The elevated levels of Hsp27 may reflect a compensatory effect after prolonged stressful conditions of polyglutamine cytotoxicity. It is consistent with a previous report which shows that 17% of the ataxin-3-positive NIs colocalized to Hsp27-positive nuclear aggregates in pontine neurons of SCA3 patients [38]. The survived cells, with increased expression of Hsp27 after prolonged stressful condition, still cannot reverse the global dysfunction of cellular proteins due to accumulation of cytotoxic effects. Therefore, a program for cell suicide (apoptosis) may eventually be initiated. It is also noted that in different passages of SK-N-SH-MJD78 cells, mutant ataxin-3 is constantly expressed (Fig. 1). Therefore, it seems likely that the increase of Hsp27 levels may not reflect a direct effect of mutant ataxin-3, but rather reflects a dynamic process of cells to cope with the stressful condition that is caused by polyglutamine cytotoxicity. It is noted that only the expression of Hsp27 is induced during the continuous cell culture process, compared to the intact expression of Hsp70 in different passages of cells (Fig. 3A). This observation suggests that Hsp27 may be a better sensor to polyglutamine cytotoxicity in neuronal cells.

Because of the late onset of polyglutamine diseases, aging may be associated with a decrease in the ability of mutant cells to cope with intracellular or environmental challenges [41,42]. The continuous culture of SK-N-SH-MJD78 cells to over 100 passages presents a cellular environment with the aggregation of ataxin-3 (Fig. 2), that is

the hallmark in the late stage of MJD. Therefore, we investigated the expression of Hsp27 under the heat shock treatment to understand whether there is attenuation in the HS response in different passages of the mutant cells. However, our results showed no significant attenuation in the response towards heat shock stress in cells carrying mutant ataxin-3 for over passage 140 (Fig. 7). The expression of Hsp27 from different passages of mutant cells showed a similar increase at 5 h after heat shock and remained at the plateau until 15 h after heat shock, followed by a gradual decrease at 20 h after heat shock. Because the induction of HSPs in response to heat shock stress is mediated largely through transcriptional activation via heat shock transcription factor 1 (HSF1) [43], our results indicated that the functions of heat shock transcription factor 1 might remain intact during the continuous cell culture process in the presence of mutant ataxin-3.

It is known that the small chaperone Hsp27 has strong anti-apoptotic properties and functions at multiple steps of the apoptotic signaling pathways [18,44–46]. The phosphorylated dimers of Hsp27 were demonstrated to interact with Daxx and prevent the interaction of Daxx with both Ask1 and Fas, therefore blocking Daxx-mediated apoptosis [47]. In addition, it is well known that heat shock proteins interact with multiple key components of signaling pathways that regulate growth and development [48]. The molecular relationships between heat shock proteins, various signaling proteins, and partner proteins appear to be critical for the normal function of signal transduction pathways. Although the functions of heat shock proteins as molecular chaperones have been well characterized, their complementary role as a ''stress-induced'' protein to monitor changes and alter the biochemical environment of the cell remains elusive. Therefore, the downregulation of Hsp27 in the early stage of MJD may trigger some irreversible damages to the neuronal cells, which cannot be repaired or reversed during the disease progression. Clearly, the effects of Hsp27 in multiple cellular pathways will have to be deciphered in order to understand which of these effects is primary in protection against neurodegeneration, which may be of great value for the development of new therapies.

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