行政院國家科學委員會專題研究計畫　成果報告

利用蛋白體學和免疫沉澱對 Machado-Joseph 疾病之探討

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(二)英文摘要及關鍵詞(keywords)。

Machado-Joseph disease (MJD)/Spinocerebellar Ataxia Type 3 (SCA3) is an autosomal dominant spinocerebellar degeneration characterized by a wide range of clinical manifestations. Unstable CAG trinucleotide repeat expansion in MJD gene has been identified as the pathologic mutation of MJD. In this study, human SK-N-SH neuroblastoma cells stably transfected with full-length MJD with 78 CAG repeats were established. Compared with parental cells, cells expressing mutant ataxin-3 displayed normal morphology for over 80 generations. Less than one percent of the transfected cells contained nuclear aggregates under basal conditions, indicating that this cellular model represented an early disease stage. While t-butyl hydroperoxide (TBH) was used to assess the oxidative tolerance of cells, the results demonstrated that the transfected cells were more susceptible to low concentrations of TBH than the parental cells. Most interestingly, from 2D gel electrophoresis analysis, we identified that the expression of heat shock protein 27 (HSP27), known as a suppressor of poly(Q) mediated cell death, dramatically decreased in SK-N-SH cells stably transfected with full-length mutant MJD. The same reduction of HSP27 was further confirmed in lymphoblastoid cells from MJD patients. However, no significant change was observed in the transcript levels of HSP27. Our results demonstrated that both neuronal and non-neuronal cells with expanded full-length ataxin-3 revealed reduced protein expression of HSP27. We proposed that the reduction of HSP27 in the disease' early stage plays an important role during cell death process in Machado-Joseph disease.

Keyword: Machado-Joseph disease, full-length mutant ataxin-3, oxidative stress, heat shock protein 27

(三)報告內容：請包括前言、研究目的、文獻探討、研究方法、結果與討論（含

結論與建議）…等。

1. INTRODUCTION

Machado-Joseph disease (MJD) belongs to a special class of inherited neurodegenerative disease caused by CAG trinucleotide repeat expansion in the coding region of the respective genes [1, 2]. In all cases, the CAG repeats are transcribed and translated into polyglutamine tracts [3]. Clinically, Machado-Joseph disease is characterized by progressive ataxia in combination with various noncerebellar symptoms, including oculomotor abnormalities, spasticity, basal ganglia symptoms, peripheral neuropathy and cognitive disturbances [4, 5]. All affected MJD patients exhibit expanded CAG's with 55 to 84 repeats whereas normal individuals exhibit 13 to 51 repeats [6]. Polyglutamine diseases are dominantly inherited, typically late-onset, fatal neurodegenerative disorders. The protein is widely expressed in neurons [7] and outside the central nervous system (CNS), but the mutation ultimately leads to selective neuronal loss in restricted brain regions. The nature of the toxic insult of a poly(Q) mutation and its biological consequences in each disease are unclear. It is possible that the poly(Q) expansion interferes with basic cellular process such as transcription, protein degradation and survival/death signaling [8]. It was shown that the ataxin-3 accumulated in ubiquitinated intranuclear inclusions selectively in neurons of affected brain regions [9]. Neuronal intranuclear inclusions have become the neuropathological sign of the CAG repeat diseases, but their cytotoxicity still remained controversy [10].

The mechanism that leads the polyglutamine-expanded proteins to aggregate is unknown. There is a possibility that the extended polyglutamine tract may destabilize the protein to misfold and aggregate. Indeed, in SCA3 brain, heat shock protein 40 (HSP 40) and HSP70 were found to localize to nuclear inclusions (NIs)[11]. In addition, different HSPs have been shown to directly inhibit several types of cell

death pathways induced by a variety of toxic insults in neuronal cells [12, 13, 14, 15]. HSP27 was reported to have anti-apoptotic properties in neuronal survival [16, 17]. 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, chemotherapeutic agents, and cytokines [18, 19, 20, 21]. 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 [22], Huntington disease (HD) and other late-onset neurodegenerative conditions [23]. Recently, it was reported that over-expression of HSP27 prevented cellular polyglutamine toxicity and suppressed the increase levels of cellular reactive oxygen species (ROS) caused by huntingtin [24]. However, the mechanisms of how HSP27 involved in the pathogenesis of Machado-Joseph disease remained unclear.

In cells transiently transfected with expanded ataxin-3 and in human SCA3 brain tissue, transcription factors CBP (cAMP response element-binding protein (CREB)-binding protein) and TBP (TATA-binding protein) were recruited into NIs, pointing to a direct interaction of the expanded ataxin-3 with specific transcription factors [25, 26]. In addition, rate mesencephalic CSM14.1 cells stably expressing expanded ataxin-3 resulted in the up-regulation of some inflammatory genes in the disease late stage [27]. However, to our best knowledge, no human neuronal cells expressing full-length mutant ataxin-3 were previously reported. In the present study, we established SK-N-SH neuroblastoma cells stably expressing HA-tagged full-length MJD with 78 CAG repeats to examine the effects of expanded ataxin-3 under normal conditions and oxidative stress. Our results showed that only less than one percent of the mutant cells contained nuclear aggregates under basal growth conditions. Stably transfected cells were more sensitive to low concentrations of TBH, indicating that transfected cells were more susceptible to the toxic insult than cells

without mutant ataxin-3. Most interestingly, we demonstrated that the protein levels of HSP27 dramatically decreased in cells with expanded ataxin-3, which may significantly impair the protection ability of the cells to respond to stress and ultimately lead to stress-induced cell death.

RESULTS and DISCUSSION

In the present study, neuronal SK-N-SH-MJD78 cells, stably expressing expanded ataxin-3, were established to analyze the role(s) of mutant ataxin-3 in human neuronal cells. Previous studies have demonstrated that neuronal cells stably transfected to express polyglutamine expansion can be established [28]. In contrast to transient transfection or inducible polyglutamine expression, cells in the present study did not undergo a rapid form of cell death under basal conditions. The clonal cells expressing expanded full-length ataxin-3 showed less than 1% intranuclear aggregates, indicating that the physiological condition of our cellular model was more likely to mimic the early stage of this late-onset disease. Therefore, this cellular model provided us a valuable system to investigate the early effects of expanded ataxin-3 in human neuronal cells.

Although no increased cell death was observed in the SK-N-SH-MJD78 cells for more than 80 passages, we could not rule out the possibility that cells may have compromised viability. Many mammalian cellular models of poly(Q) disease have shown that overexpression of poly(Q) protein containing a poly(Q) expansion resulted in toxicity/cell death [24, 28]. Chemicals and conditions that damage proteins, promote protein misfolding, or inhibit protein processing triggered the onset of protective homeostatic mechanisms resulting in "stress responses" in mammalian cells. In the present study, we were interested in how neuronal cells containing expanded ataxin-3 respond to external oxidative stress. It was reported that rat

pheochromocytoma PC12 cells stably expressing polyglutamine expansion were more vulnerable to exogenous stress [28]. To test the hypotheses that oxidative stress is indeed a contributory factor for the pathogenesis of Machado-Joseph disease, we exposed cells to oxidative stress by the use of low concentrations of TBH. Our results demonstrated that cells with expanded ataxin-3 were more susceptible to exogenous oxidative stress than the parental cells, indicating that these cells had weak protection effects upon the oxidative stress. Our results demonstrated that the expression of expanded ataxin-3, even though it did not dramatically elevate protein aggregate formation under basal conditions, did impair the ability of the cell viability to respond to extracellular oxidative stress.

In an attempt to determine whether the expression of expanded ataxin-3 altered gene expression that may be important in the neurotoxicity observed in Machado-Joseph disease, comparative proteome analysis was performed using the total protein extract from cells with and without expanded ataxin-3. Most interestingly, a dramatic expression difference in HSP27 was identified via two-dimensional gel electrophoresis followed by Tandem Mass analysis. Western blot analysis demonstrated that the expression of HSP27 in transfected cells retained only 20% of that of the parental cells. The same reduction of HSP27 was further confirmed in two lymphoblastoid cell lines derived from MJD affected individuals, when compared with that from the normal control. It ruled out the possibility that the reduction of HSP27 was the result from over-expression of a certain protein in SK-N-SH cells. In addition, the reduction of HSP27 was observed by immunocytochemical staining, which showed very significant alterations in cells with and without expanded ataxin-3. It was worthy noting that the reduced HSP27 expression was not due to transcriptional dysregulation, as indicated through semi-quantitative RT-PCR and microarray analysis. There were no significant alterations in HSP27 mRNA levels

from cells with or without expanded ataxin-3. This observation ruled out the possibility that HSP27 gene may be disrupted by the transgene in our stable cell line. It was shown that HSP27 was regulated at different levels along with the expression, including transcriptional, translational and posttranslational [29]. Further studies will be required to address the mechanism underlying the reduced protein level of HSP27 in the disease model.

HSP27 was reported to protect cells against oxidative stress and have anti-apoptotic properties in neuronal survival [16, 17, 24, 30]. The expression of HSP27 led to a decrease in reactive oxygen species (ROS) and to an increase in glutathione [30, 31]. Recently, it was reported that mutant huntingtin caused increased levels of ROS in neuronal cells and transiently transfected HSP27 suppressed the increased levels of cellular ROS without interfering poly(Q) aggregation [24]. Therefore, it is possible that a reduction of HSP27 in the presence of expanded ataxin-3 resulted in an increase of ROS in the cellular model. However, we cannot rule out the possibility that the reduced level of HSP27 may be involved in other pathway(s) that lead to cell death in the pathogenesis of MJD. It was reported that HSP27 inhibits the mitochondrial death pathway by binding to and inhibiting apoptosome formation [13]. In addition, 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 [32]. It is possible that HSP27 has a specific target in a key apoptotic signaling or execution pathway, which was supported earlier by the report that HSP27 can prevent activation of pro-caspase 9 after etoposide treatment and can inhibit apoptosis induced by activation of the Fas receptor [30, 33]. Our next works will be addressing which pathway that HSP27 may be involved in the molecular pathogenesis of MJD.

Even though we do not yet understand the mechanism responsible for the

significant reduction of HSP27, this observation in the early disease stage suggested that the loss of HSP27 protection activity together along with aging might ultimately lead to cell death. However, we cannot exclude the possibility that other cellular proteins involving signal transduction and/or apoptosis pathways also play important roles in the pathogenesis of MJD. It is important to note that the neuronal cells contained the ability to withstand the existence of expanded ataxin-3 for prolonged periods without apparent adverse effects in our cellular model. It is likely that cells underwent genetic or biochemical changes that allowed them to cope with expanded full-length ataxin-3 in the disease' early stage. Further analysis of the roles of expanded ataxin-3 will help to better understand its physiological functions and how the poly(Q) expansion in the mutant protein interferes with those and/or other activities.

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(四) 計畫成果自評部份

研究內容與原計畫蛋白體學研究相符，免疫沉澱雖尚未達成預期目標情況,正進一步研究當中，此研究成果適合在學術期刊發表