行政院國家科學委員會專題研究計畫成果報告

國科會專題研究計畫成果報告撰寫格式說明

Preparation of NSC Project Reports

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一、中文摘要

Machado-Joseph Disease 簡稱 MJD，是體染色體顯性的遺傳性疾病，屬於漸進性神經退化性疾病的一種亞型。MJD 主要的臨床表徵則為運動障礙、肌肉萎縮、及錐體系路徑症狀等。調控此症狀的基因座落於第十四對染色體的長臂上(14q32.1)，稱為 MJD 基因。在此基因的 3'端轉譯區(3'-translated region)內有一段異常的 CAG 核酸重複序列發生倍增突變(amplification mutation) 是造成疾病的原因。我們近年來從臨床醫師的病歷診斷幫助，再配合實驗室分子生物診斷 MJD 的技術已臻成熟，基礎研究方面，本三年計劃研究至目前為止的主要成果，一、建立了表達突變 MJD 的神經腫瘤細胞株。二、利用 oxidative stress 討論環境壓力是否在突變的 MJD 蛋白的聚集作用上扮演必要角色，人類神經細胞較正常細胞對 t-butyl peroxide 的氧化刺激更為敏感;三、利用二維膠體電泳(2-dimension gel analysis)討論，穩定表達致病 MJD 蛋白的人類神經細胞株中，多個蛋白的表達受到影響 四、利用不同細胞程序性死亡(apoptosis) inducer 證實異常擴增 MJD 蛋白的人類神經細胞株程序性死亡，並不是藉由 Caspase 12 的路徑，而是進行 non-ER stress 的程序性死亡，五、利用微矩陣研究，穩定表達致病 MJD 蛋白的人類神經細胞株中，多個基因的表達受到影響，目前更進一步的實驗正在神經細胞中進行中。六、繼續新的 SCA 突變基因的偵測，包括 SCA12 及 SCA17。我們相信這個研究計畫的結果，將對 MJD 此遺傳性漸進神經退化性疾病致病原因及晚發性等病理現象，有進一步的了解。

關鍵詞： Machado-Joseph Disease, 神經退化性疾病, 神經腫瘤細胞株, 細胞程序性死亡, 二維膠體電泳

Abstract

Machado-Joseph disease (MJD) is an autosomal dominant spinocerebellar degeneration characterized by cerebellar ataxia and pyramidal signs associated in varying degrees with a dystonic-rigid extrapyramidal syndrome or peripheral amyotrophy as major neurologic signs. Unstable CAG trinucleotide repeat expansion in MJD gene on the long arm of chromosome 14 has been identified as the pathologic mutation for MJD. In the past few years, we have established the clinical molecular diagnosis method for the routine screening. In turns of the basic research, so far, the results of the 3-year projects are as the followings. (1) Establishment of the stable neuroblastoma cell line expressing the mutant MJD protein (2) Evaluate the roles of oxidative stress play at the mechanisms of protein aggregation of mutated ataxin-3. Mutant cells are more sensitive to t-BH treatment. (3) By the means of 2-Dimensional gel analysis, several genes' expression levels are either up- or down regulated. (4) Take advantage of the various apoptosis inducers, we demonstrated that cells undergo non-ER stress apoptosis in the presence of the MJD mutant protein. (5) The results from microarray analysis demonstrated that a few genes including elongation factors, transcription factors and phosphatase are up-regulated in the presence of MJD protein. (6) We also established the distributions of the repeat number in SCA12 and SCA17 in the normal populations in Taiwan. All the above experiments are under way and some preliminary results are obtained.

Keywords: Machado-Joseph Disease,

neurodegenerative disease, neuroblastoma cells, apoptosis, 2-dimensional gel

二、緣由與目的

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, in all cases, the CAG repeats are transcribed and translated into polyglutamine tracts. Diseases in this class include Huntington's disease (HD)(The Huntington's disease collaborative research group, 1993), spinocerebellar ataxia type 1 (SCA 1) (Orr et al., 1993), spinal and muscular atrophy (SBMA or Kennedy disease) (La Spada et al., 1992), Machado-Joseph disease (MJD) (Kawaguchi et al., 1994), dentatorubral-pallindoluysian atrophy (DRPLA) (Koide et al., 1994; Nagafuchi et al., 1994), spinocerebellar ataxia type 2 (SCA 2) (Pulst et al., 1996; Sanpei et al., 1996; Imbert et al., 1996), spinocerebellar ataxia type 6 (Zhuchenko et al., 1997), spinocerebellar ataxia type 7 (David et al., 1997) and spinocerebellar ataxia type 17 (Nakamura et al., 2001). 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 (Burk et al., 1996; Durr et al., 1996). All affected MJD patients exhibit expanded CAG's with 55 to 84 repeats whereas in normal individuals exhibit 13 to 51 repeats. Inverse correlation between the expanded repeat length and the age-at-onset of the trinucleotide disease has been reported in HD (Andrew et al., 1993; Duyao et al., 1993; Snell et al., 1993), DRPLA (Koide et al., 1994), SCA 1 (Jodice et al., 1994; Ranum et al., 1994), SBMA (La Spada et al., 1992), MJD (Maruyama et al., 1995; Maciel et al., 1995) and SCA 2 (Pulst et al., 1996; Sanpei et al., 1996; Imbert et al., 1996). In addition, the trinucleotide repeat mutation in most of these diseases is associated with the phenomenon of anticipation, where the disease tends to present at an earlier age and more severe clinical manifestations in successive generations. Polyglutamine diseases are dominantly inherited, typically late-onset, fatal neurodegenerative disorders. In addition, it was reported that roughly equal levels of normal and expanded repeat ataxin-3 through immunohistochemical method, there is no evidence to link between the disease' late-onset phenomena to the ataxin-3 protein, either normal or expanded mutant. The protein is widely expressed in neurons (Gutekunst et al., 1999) and outside the CNS, but the mutation ultimately leads to selective neuronal loss in restricted brain regions. It was shown that the ataxin-3 accumulates in ubiquitinated intranuclear inclusions selectively in neurons of affected brain regions (Paulson et al., 1997). It is suggested that intranuclear aggregation of the expanded protein may be initiated or catalyzed by a glutamine-containing fragment of the disease protein. Peoteolytic cleavage has been implicated by the findings of truncated polyglutamine fragments in inclusions. Neuronal intranuclear inclusions have become the neuropathological sign of the CAG repeat diseases, but their cytotoxicity still remains controversy (Yamada et al., 2000). In cells transiently transfected with expanded ataxin-3 and in human SCA3 disease tissue, the transcription factors CBP (camp response element-binding protein (CREB)-binding protein) and TBP (TATA-binding protein) are recruited into NIs, pointing to a direct interaction of the expanded disease proteins with specific transcription factors (Perez et al., 1998; McCampbell et al., 2000). Furthermore, it was shown that normal and expanded full-length ataxin-3 associated with the nuclear matrix and adopted a novel conformation, probably enabling interaction with nuclear proteins (Tait et al., 1998; Perez et al., 1999). Together, these studies strongly suggest that the expanded disease proteins in CAG repeat disorders cause transcription dysregulation. However, the pathogenesis and molecular basis of neuronal cell death in Machado-Joseph disease remain unclear.

Oxidative stress is induced by reactive oxygen species (ROS) or free radicals, and has been proposed to play an important role in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease (Beal, 1995). Oxidative stress causes modification of cellular macromolecules and leads to cell damage. Protein is susceptible to attack in the presence of reactive oxygen species. Several types of protein damage have been identified, including amino acid modification, cross-linking, and protein degradation (Schuessler and Schilling, 1984; Dean

et al., 1991). One of these modifications, thiol groups in a cysteine residue is one of the spots most likely to be modified and, then cause cell damage. The mixed disulfide formation between protein thiols and GSH has been demonstrated in human red blood cells under oxidative stress (Lii and Hung, 1997). Here, we try to identify protein modifications that relate to thiol groups in human neuroblastoma cells with and without the expression of MJD protein under oxidative stress. Bcl-2 protects cells against diverse variety of cytotoxic insults such as radiation, dexamethasone, staurosporine and withdrawal of cytokines and growth factors (Blagosklonny et al., 2001). Apoptosis is important for development and tissue homeostasis, but too much or too little apoptosis can also cause disease (Los et al., 1999; Friedland and Yuan, 1998). According to the biochemical definition, apoptosis is a cell death mediated by caspases (Fadeel et al., 2000; Blagosklonny et al., 2000). So far, 14 family members of caspases have been identified (Nakagawa et al., 2000). In particular, caspase-9 is activated in the mitochondria-dependent pathway involving cytochrome C and Apaf-1. Also, caspase-8 can indirectly activate caspase-9. Caspase-9 activates executioner caspase-3, caspase-6 and caspase-7. Bcl-2 prevents caspase-9 activation, the major apoptotic pathway activated by cytotoxic stimuli. When caspase-9 plays a crucial role in cell death, overexpression of Bcl-2 can prevent apoptosis (Green and Reed, 1998). Because Bcl-2 acts upstream of caspases, it does not significantly affect caspase-independent cell death. Although Bcl-2 prevents apoptosis, it only delay cell death (Yin et al., 1995). Previously, experiments performed in primary strialtal neurons showed that the polyglutamine cytotoxicity can be inhibited by the anti-apoptotic protein Bcl-X1 and caspase inhibitors (Sauou et al., 1998). Involvement of caspases is also indicated in SCA1 transgenic mice model (Ona et al., 1999) and in HD lymphoblasts (Sawa et al., 1999). Stress in the endoplasmic reticulum (ER) can also result in apoptosis (Welihinda et al., 1999; Nakagawa et al., 2000). It was shown that caspase-12 is localized to the ER and activated by ER stress, including disruption of ER calcium homeostasis and accumulation of excess proteins in ER. Caspase-12 mediates an ER-specific apoptosis pathway and may contribute to amyloid-β neurotoxicity (Nakagawa et al., 2000). However, much less is known about the proteolysis pathway and the role of caspase(s) may play in the pathogenesis of Machado-Joseph disease.

In the present study, we utilized neural SK-N-SH cells stably transfected with HA-tagged full-length MJD with 78 polyglutamine repeats to examine the effects of polyglutamine expansion on protein aggregation and neural cell survival under different environmental stress. Our result showed that less than one percent of the mutant cells containing nuclear aggregates under basal condition. In addition, t-butyl hydroperoxide and various pro-apoptotic agents were used to assess the tolerance of the mutant cells. Cell Viability was determined by MTS assay. Mutant MJD cells were more sensitive to low concentration of t-butyl hydroperoxide (1 μM to 3 μM) than the normal cells without expanded MJD. We demonstrated that the ratio of GSH/GSSG increased about 4-fold in mutant MJD cells and the enzyme activities of Glutathione peroxidase and Glutathione reductase remained only 40% of those of the normal cells after 12 hours treatment of 1 μM TBH. On the other hand, mutant MJD cells treated with 1 μM staurosporine showed significant decreased Bcl-2 level when compared to the normal cells. It is consistent to our observation that cells expressing mutant MJD were more sensitive to staurosporine-induced apoptosis. Taken together, MJD cells are more susceptible to these toxic insults than the normal cells without expanded MJD. We demonstrated that expression of full-length mutant MJD does not dramatically elevate protein aggregate formation under basal conditions, but does significantly impair the ability of the cell viability to respond to stress and alter the redox status of the cellular proteins, which may lead to increase stress-induced cell death following stress.

三、結果與討論

Results

Expression of mutant MJD in SK-N-SH cells

SK-N-SH cells were stably transfected with full-length poly-Q expanded MJD construct (pCDNA3-MJD78), resulting SK-N-SH-MJD78. Both SK-N-SH and SK-N-SH-MJD78 showed similar morphology. Expression of the exogenous proteins in the different subclones was confirmed by Western blot analysis and immunocytochemistry using antibodies against MJDor against HA epitope because the

pCDNA3-MJD78 constructs are HA-tagged. Size and stability of the exogenously expressed proteins over time was confirmed on lystaes obtained from cells at the 20^{th}, 40^{th} and 80^{th} passages, respectively. Most of the expressed ataxin-3 is found in the cytoplasm. Only less than one percent of the cells showed nuclear inclusions from the observation.

WT and mutant MJD differentially affect cell viability upon the t-butyl hydroxide treatment

While SK-N-SH cells were exposed in vitro to t-butyl hydroperoxide from 1 μM to 3 μM, increased cell death was observed in cells expressing the mutant MJD compared to its parental cells, SK-N-SH. Quantitations of these effects by MTS assays, which measures cell viability, confirmed that all stable clones behaved approximately the same in normal growth conditions. However, when challenged by 1 μM or 3 μM t-butyl hydroperoxide, significant differences in cell viability were observed between cells with and without mutant MJD proteins. Cultures of parental SK-N-SH cells did not exhibit a dose-dependent decrease in cell viability by MTS assay after 1 and 3 μM t-butyl hydroxide. However, this decrease was evident in SK-N-SH-MJD78 cells. To determine if the cell death observed in the presence of mutant MJD is attributable to the change of activities of certain antioxidant enzymes, we checked the oxidized (GSSG) and the reduced glutathione (GSH) through the ratio of GSH/GSSG and assayed antioxidant enzyme activities of Glutathione peroxidase (GSH-px) and reductase (GSSG-R). The results showed that the ratio of GSH/GSSG increased about 4-fold in mutant MJD cells when compared to that of the cells harboring the normal MJD proteins. While the enzyme activities of Glutathione peroxidase and Glutathione reductase remained only 40% of those of the normal cells after 12 hours treatment of 1 μM TBH.

Mutant MJD induces non-ER stress apoptotic cell death

To determine whether the caspase-12 pathway involved in the pathogenesis of MJD, we examines cell viability in response to different apoptotic stimuli. SK-N-SH cells and SK-N-SH-MJD78 cells were first treated with brefeldin A and tunicamycin, which can induce effective cleavage of procaspase-12, and then assayed by MTS assay. The results indicated no significant differences between cells with and without mutant MJD in response to up to 10 μg/ml of brefeldin A or tunicamycin for up to 96 hours. On the other hand, SK-N-SH cells and SK-N-SH-MJD78 cells were then treated with staurosporine, a non-ER stress inducing apoptotic stimuli. Mutant MJD expression cells were more sensitive to the non-ER stimuli, as evidenced by more dramatic decrease of cell viability under the treatment of 1 μM of staurosporine. The results indicated that caspase-12 may not directly involve in the pathogenesis of MJD.

Decreased Bcl-2 expression in cells containing mutant MJD proteins

On the other hand, mutant MJD cells treated with 1 μM staurosporine showed significant decreased Bcl-2 level when compared to the normal cells. It is consistent to our observation that cells expressing mutant MJD were more sensitive to staurosporine-induced apoptosis.

Discussion

In the present study we utilized neural SK-N-SH-MJD78 cells that have been stably transfected with polyglutamine expansion constructs, in order to analyze aggregate formation and mutant MJD's role upon oxidative stress and apoptotic stimuli. Previous studies have demonstrated that neural cell lines stably transfected to express polyglutamine expansion can be established (Li et al., 1999). In contrast to transient transfection or inducible polyglutamine expression, cells in the present study do not undergo a rapid form of cell death under basal conditions, and have thus have likely undergone genetic or biochemical changes which allow them to cope with polyglutamine expansion. It is interesting to note that the ability of neuronal cells to withstand the existence of MJD with polyglutamine expansion for prolonged periods without apparent adverse effects in our cellular model. However, it is also important to note that cells stably expressing polyglutamine expansion have been demonstrated to be Polyglutamine, aggregation, proteasome, and neural survival more vulnerable to stress (Li et al., 1999), and were more prone to aggregate formation in the present study. Very few of the cells, less than one percent

of the observed cells, in the present study demonstrated nuclear aggregates (Fig 3C), possibly indicating that aggregation within the nucleus may be particularly important in the initiation of cytotoxicity. Although no increase in cell death was observed following more than 80 passages, data in the present study do not rule out the possibility that cells may have compromised viability. It will be important in future studies to determine if MJD protein containing expanded polyglutamine alters gene expression or signal transduction pathways that may be important in the neurotoxicity observed in polyglutamine disorders. We are interested in how cells containing mutant MJD cells response to genetic and environmental stress. It was reported that three suppressors that identify genes and pathways not previously known to be involved in polyglutamine-induced neurodegeneration, including glutathione-S-transferase (GST) gene (Fernandez-Funez et al., 2000). These findings implicate oxidative and/or chemical stress in polyglutamine neurodegeration. It is known that the brain also has a cellular defense system against oxidative stress. This system includes high levels of several antioxidant enzymes (e.g. catalse, SOD, GSH-px, and GSSG-R) (Lee et al., 2000). GSH and GSH-px play predominant roles in removing excess H_2O_2 in the brain (Freeman and Crapo, 1982; Sampath et al., 1994). However, little is known about the cellular distribution of antioxidants and antioxidant enzyme in the CNS. In the present study, we demonstrate that cells expressing mutant MJD proteins change the balance of the antioxidant enzymes in the cellular model. In our system, SK-N-SH-MJD78 cells contain low concentrations of antioxidant enzymes, suggestion that these cells have weak protection effect upon the oxidative stress. In addition, it was demonstrated that regulation of mitochondrial and/or cytosolic reactive oxygen intermediates (ROI) levels could be mediated, in part, by the Bcl-2 gene product (Hockenbery et al., 1990). Expression of the Bcl-2 protein prevents the induction of apoptosis by a variety of oxidative stresses, including inhibition of GSH synthesis, ionizing radiation, and heat shock (Zhong et al., 1993; Hockenbery et al., 1990). It has been suggested that the Bcl-2 gene product inhibits apoptosis by interacting with mitochondrial superoxide dismutase (SOD) (Itoh et al., 1993). Therefore, we were interested in the expression levels of Bcl-2 in the cells with and without mutant MJD. As expected, mutant MJD cells treated with 1 μM staurosporine showed significant decreased Bcl-2 level when compared to the normal cells. It is consistent to our observation that cells expressing mutant MJD were more sensitive to staurosporine-induced apoptosis. Taken together, MJD cells are more susceptible to these toxic insults than the normal cells without expanded MJD. We demonstrated that expression of full-length mutant MJD does not dramatically elevate protein aggregate formation under basal conditions, but does significantly impair the ability of the cell viability to respond to stress and alter the redox status of the cellular proteins, which may lead to increase stress-induced cell death following stress.

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四、 計畫成果自評

主持人認為研究成果內容已達成相當的預期目標並可作為後續研究之用。神經細胞系統實驗的結果將具有疾病早期影響的代表性意義。此研究成果將具相當學術價值適合在學術期刊發表。目前實驗及正式論文正積極進行中。

附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※Machado-Joseph 疾病致病病因之探討 ※

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學生命科學系

中 華 民 國 91 年 10 月 20 日