附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

碳酸酐酶相關蛋白與 Bcl-2 基因調控在小腦脊髓運動失調症之角色研究
Studies of gene regulation of carbonic anhydrase-related proteins and Bcl-2 in spinocerebellar ataxias

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 94－2311－B－029－006－
執行期間：　94 年 08 月 01 日至　95 年 07 月 31 日

計畫主持人：謝明麗
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計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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中　華　民　國　　年　　月　　日

中文摘要：

Spinocerebellar ataxia type 3 (SCA3) 或稱為 Machado-Joseph disease (MJD)，為晚發性神經退化性疾病。其造成神經退化的機制為所表達的 ataxin-3，含有一段多麩醯胺酸序列。本實驗室過去利用微陣列分析的結果顯示，穩定表達突變 ataxin-3 的 SK-N-SH-MJD78 細胞株，與正常 SK-N-SH 細胞株比較下，可發現碳酸酐腖相關蛋白-11 (CA-RP-11)於 SK-N-SH-MJD78 細胞株中有較高的表達含量。於接下來的研究中，本實驗室利用 CA-RPs 抗體做免疫染色相關實驗發現，CA-RP-8 及 11 於穩定表達突變 ataxin-3 的神經細胞中，比起表達正常 ataxin-3 的神經細胞，有較高的表達含量。半定量反轉錄聚合酶酵素反應 (semi-quantitative RT-PCR)實驗證明，CA-RP-8，10 及 11 於表達突變 ataxin-3 的神經細胞中，在轉錄層次上的表達含量皆明顯上升。當神經細胞短暫表達突變 ataxin-3 時，也可發現 CA-RP-11 表達含量有升高趨勢。我們更發現，當細胞表達突變 ataxin-3 時，CA-RP-11 不但於核外細胞質中表達，甚至有轉移至核內表達的情況。於 MJD 轉殖基因老鼠之鼠腦及疾病人腦組織中，仍可發現 CA-RP-11 在腦細胞的細胞質及細胞核，也有高度表達現象。於核內堆積物中含有 CA-RP-11 及突變 ataxin-3，可能顯示 CA-RP-11 於疾病進程中，可能扮演某種關係。雖然在中樞神經系統中，碳酸酐腖相關蛋白之確切功能仍然不清楚，但是，於本實驗室研究發現，碳酸酐腖相關蛋白，特別是 CA-RP-11，可能於 MJD 疾病中，扮演著重要角色。

關鍵字：第三型小腦脊隨萎縮症、突變 ataxin-3、SK-N-SH、碳酸酐腖相關蛋白 (CA-RPs)

Abstract

Spinocerebellar ataxia type 3 (SCA3) is a late-onset neurodegenerative disorder caused by the expansion of a polyglutamine tract within the gene product, ataxin-3. Microarray analysis revealed a dramatic differential expression of carbonic anhydrase-related protein XI in the presence or absence of mutant ataxin-3. Therefore, we examined the expression and distributions of CA-RP VIII, X and XI in human neuronal cells stably expressing mutant ataxin-3. The protein expression of CA-RP VIII and CA-RP XI is significantly increased in human neuroblastoma cells containing mutant ataxin-3, compared to cells containing normal ataxin-3. Semi-quantitative RT-PCR demonstrated that all three CA-RPs exhibited significantly higher transcript levels in neuronal cells expressing mutant ataxin-3. Results from transient transfection assays also confirmed the up-regulation of CA-RPXI in mutant ataxin-3 transfected cells. Interestingly, CA-RP XI is distributed not only in cytoplasm but also translocated within the nucleus of the stably transfected mutant cells, compared to the cytoplasm distribution in cells containing normal ataxin-3. Most importantly, immunohistochemical staining of the MJD transgenic mice and postmortem MJD human brain also revealed that CA-RP XI highly expresses in both cytoplasm and nucleus of the brain cells. Recruitment of CA-RPXI into nuclear inclusions containing mutant ataxin-3 revealed a possible correlation between CA-RPXI and disease progression. Although the exact function of CA-RPs is still undefined in the central nervous system, our findings suggest that CA-RPs, especially CA-RP XI, may play specific roles in the pathophysiology of Machado-Joseph disease.

Keyword: Spinocerebellar ataxia type 3, Mutant ataxin-3, SK-N-SH, CA-RPs

Introduction and purpose

Spinocerebellar ataxia type 3 (SCA3) or Machado-Joseph disease (MJD) is an autosomal dominantly inherited neurodegenerative disorder with a wide range of clinical manifestations, including ataxia, ophthalmoplegia, pyramidal signs, basal ganglia symptoms, and peripheral neuropathy. The mutation causing SCA3 is an unstable CAG trinucleotide repeat expansion within exon 10 of a gene encoding ataxin-3 (Kawaguchi et al., 1994). All affected SCA3 patients exhibit expanded CAG's with 55 to 84 repeats whereas normal individuals exhibit 13 to 51 repeats (van Alfen et al., 2001). 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 (Yuasa et al., 1986). The nature of the toxic insult of a polyQ mutation and its biological consequences for the disease are still unclear. Several studies have demonstrated that protein fragments containing an expanded polyglutamine possess an increased vulnerability to apoptotic death. However, the mechanisms underlying the slow cell death processes are largely unknown. Genetic and molecular studies have suggested that polyQ causes altered gene expression, abnormal protein interactions, alteration of proteolysis, and activation of caspases and protein unfolding (Paulson et al., 2000; McCampbell and Fischbeck, 2001; Nucifora et al., 2001; Dunah et al., 2002; Wen et al., 2003; Chang et al., 2005; Li et al., 2002; Evert et al., 2003). Furthermore, 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 (Paulson et al., 1997). Neuronal intranuclear inclusions have become the neuropathological sign at the late stage of the triplet diseases, but the relationship between aggregation and cytotoxicity remains controversial (Yamada et al., 2000; Tobin and Signer, 2000).

In order to understand the putative gene expression alteration(s) in the presence of mutant ataxin-3, microarray analysis was performed by the use of RNAs extracted from human neuroblastoma cells SK-N-SH and SK-N-SH-MJD78, stably expressed mutant ataxin-3 (Wen et al., 2003). From microarray analysis, the expression of carbonic anhydrase-related protein XI showed significant alteration in the presence of mutant ataxin-3. The α-carbonic anhydrases (CAs) (EC 4.2.1.1) constitute a family of monomeric zinc metalloenzymes that catalyze the reversible hydration of CO_2 (Sly and Hu, 1995). To date, 12 human isozymes exhibiting the characteristic enzyme activity of CA have been reported (see review, Hewett-Emmett, 2000, Lehtonen et al., 2004). Along with "active" CA isozymes, evolutionally conserved but "acatalytic" family members have been reported and designated carbonic anhydrase-related proteins (CA-RPs): CA-RPs VIII, X, and XI (Tashian et al., 2000). CA-RPs lack one or more

histidine residues required to bind the zinc iron, which is essential for CO_2 hydration activity, and thus are believed to be inactive with regard to classical CA activity (Hewett-Emmett and Tashian, 1996). The exact biological function of these CA-RPs have not been elucidated (Tashian et al., 2000). Immunohistochemical analysis showed that CA-RPs VIII and XI are consistently expressed in neural cells, astrocytes, and neurites of most parts of human and mouse brains (Taniuchi et al., 2002 a and b). CA-RP X is expressed in both the myelin sheath and neural cells. Furthermore, it was demonstrated recently that CA-RP VIII deficiency is associated with a distinctive lifelong gait disorder in Waddles mice, suggesting CA-RP VIII plays a central role in motor control (Jiao et al., 2005). However, not only the biological functions but also the subcellular distributions of these inactive CA isozymes are still ill-defined.

In order to understand the role of CA-RPs in the pathophysiology of Machado-Joseph disease (MJD), we used the neuron cellular model, the brain tissue of MJD transgenic mice and postmortem MJD human to observe the expression of CA-RPs.

Methods

1. Plasmid constructs: pEGFP-N1-MJD26, pEGFP-N1-MJD78 and pEGFP-N1-trunMJD78
2. Generation of stably transfected MJD cells
3. Comparative analysis for mRNA expression of CA-RPs by RT-PCR
4. Immunocytochemical staining
5. Immunoprecipitation and immunoblotting

Results and discussion

The mRNA expressions of three CA-RPs were studied by RT-PCR with varying PCR cycles in cells expressing normal or mutant ataxin-3. cells expressing normal ataxin-3 showed lower expression levels of all three CA-RPs than cells expressing mutant ataxin-3. hese findings exhibited significant higher transcript levels of all three CA-RPs in neuronal cells expressing mutant ataxin-3.

To study the cellular distributions of all three CA-RPs in the human neuroblastoma cells with and without mutant ataxin-3, we used monoclonal antibodies to each CA-RP isozyme. we showed perinuclear and membranous distribution of CA-RPVIII in the parental SK-N-SH cells

without ataxin-3 mutation. Previously, CA-RP VIII was reported to bind inositol (1,4,5)-triphosphate (IP_3) receptor type I (IP_3R1) and to inhibit IP_3 binding to IP3R1 by reducing the affinity of the receptor for IP_3 (Hirota et al., 2003). In our study, the perinuclear localization of CA-RP VIII observed in SK-N-SH cells may suggest its co-localization with IP_3R1, which is known to be expressed on the membrane of the endoplasmic reticulum. The inositol (1,4,5)-triphosphate receptor (IP_3R) is an intracellular calcium (Ca^{2+}) release channel that plays an important role in neuronal Ca^{2+} signaling (Berridge,1998). The exact reason of the more intense staining and evenly distributed of CA-RP VIII in the cytoplasm of SK-N-SH-MJD78 cells is still unknown. Previous studies of Huntington's disease (HD), caused by polyglutamine expansion (exp) in huntingtin (Htt), revealed that hunthingtin (Htt) and huntingtin-associated protein 1A (HAP-1A) influence neuronal calcium signaling mediated by IP_3R1 (Tang et al., 2003). It was demonstrated that IP_3R1-HAP1A-Htt ternary complex is formed in vitro and in vivo. IP_3R1 activation by IP_3 is sensitized by Httexp, but not by normal Htt. Therefore, our results indicated that the expression and localization of CA-RP VIII are influenced by the presence of mutant ataxin-3, suggesting a possible link between mutant ataxin-3 and IP_3R1-mediated neuronal Ca^{2+} signaling via regulation of CA-RP VIII.

Compared to the parental SK-N-SH cells or SK-N-SH stably expressing normal ataxin-3, the expression levels and cellular localizations of CA-RP XI were altered significantly in the cells stably expressing mutant ataxin-3, as evidenced by semi-quantitative RT-PCR, immunocytostaining, and immunofluorescence. Cellular localization of CA-RP XI is redistributed to not only cytoplasm but also nuclei of cultured neuroblastoma cells which are stably expressing mutant ataxin-3, compared to the pure cytoplasmic distribution in cells expressing normal ataxin-3. To confirm our findings from cellular environment in MJD transgenic mouse model, immunohistochemical staining was performed in the control and MJD transgenic mouse brain sections. The most notable finding was the intense and nuclear localization of CA-RP XI in MJD transgenic mouse brain sections. The antibody to CA-RP XI clearly demonstrated nuclear localization in different regions of SCA3 transgenic mouse brain sections. Additionally, immunostaining of post-mortem SCA3 brain (cerebellar) tissues also confirmed this observation. Taken together, the abovementioned results indicated that CA-RP XI, expressed in the cytoplasm of the cells expressing normal ataxin-3, changes its cellular localization and expression level in the presence of mutant ataxin-3.

It is well-known that the nuclear inclusions in MJD are the hallmark of the late stage of the disease. 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 (Paulson et al., 1997). Therefore,

whether there is direct interaction between CA-RP XI and mutant ataxin-3 was also examined in this study. Up to date, the functions of CA-RPs in neuronal cells are still ill-defined, we cannot rule out the possibility that the redistribution of CA-RP XI to the nuclei of neuronal cells may play critical role(s) in the disease progression. However, further investigations will be needed before reaching conclusions.

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