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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※　進行性肌陣攣性癲癇相關之 cystatin B 基因的表現　※
※　　　　　　　對於神經細胞凋亡之影響　　　　　　　※
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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC90－2314－B－038－040－

執行期間：　90 年 8 月 1 日　至　91 年 7 月 31 日

計畫主持人：陳怡如

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□國際合作研究計畫國外研究報告書一份

執行單位：臺北醫學大學 醫學系小兒學科

中　華　民　國　　91 年　10 月　15 日

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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執行期限：90 年 8 月 1 日至 91 年 7 月 31 日

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中文摘要

Cystatin B 是一種廣泛存在於各種組織的蛋白質，其含有 98 個氨基酸，會抑制多種硫胱氨酸蛋白酵素的作用，包括 cathepsins L、H、B、S 及木瓜蛋白酵素。Cystatin B 基因的突變導致人類第一型進行性肌陣攣性癲癇，而缺少 cystatin B 的基因轉殖鼠也發展出類似症狀，且小腦顆粒細胞的的凋亡增加。缺乏 cystatin B 表現對於中樞神經細胞凋亡促進的原因尚不清楚。由於其它研究顯示 cathepsin B、D 及 L 與神經細胞的凋亡有關，有人認為 cystatin B 含量的降低會導致 cathepsin 活性不正常提高，而活化了細胞凋亡所必需的 caspases。為了進一步了解 cystatin B 在神經細胞凋亡中所扮演的角色，本計畫中將 cystatin B 的基因的 cDNA 以正向或反向轉殖入表現載體中，並將這些殖體轉染到腎上腺親鉻母細胞瘤細胞株 PC12 中，挑選 stable trasfected cell clones，藉以提高或是抑制 cystatin B 的表現。由於 PC12 細胞在神經生長激素 NGF 的刺激後會分化成神經細胞，這些 cystatin B 表現量不同的 PC12 細胞株將可用來研究該蛋白對於未分化 PC12，以及及受 NGF 刺激分化之 PC12 細胞之細胞凋亡的影響。未來還可進一步用來研究 cystatin B 蛋白與細胞凋亡相關的機制。

關鍵詞：PC12、cystatin B、細胞凋亡、癲癇。

ABSTRACT

Cystatin B is a ubiquitously distributed small protein 98 amino acid in size, which binds and inhibits the cystein proteases including cathepsins L, H, B, S and plant cysteine protease papain. Mutations in cystatin B gene caused progressive myoclonic epilepsy (EPM1) in human, and cystatin B deficient mice represent similar symptom, with increased apoptosis in cerebellar granule cells. The mechanism by which lacking of cystatin B expression promotes apoptosis in CNS is still not clear. However, other studies showed that cathepsin B, D and L are involved in the apoptosis of serum deprived PC12 cells and hippocampus CA 1 pyramidal neuron after ischemia, and cathepsin B is involved in the processing of several procaspases. It was proposed that reduction in cystatin B might increase apoptosis by inappropriate activation of cathepsins and thereby increase the activation of caspases that are required for the apoptosis to occur. To further investigate the function of cystatin B protein in apoptosis of neuronal cells, we cloned the cDNA of cystatin B gene in either sense or antisense orientation into the pCDNA3 expression vector, transfect these plasmid into the rat adrenal pheochromocytoma PC12 cells, and select for stable transfected clones. The cell clones expressing higher (sense) or lower (antisense) level of cystatin B protein were selected for our experiments. Since the PC12 cell can be induced to neuronal differentiation by NGF, these stable trasfected cell clones can be used to study the effect of cystatin B protein on the apoptosis of undifferentiated and NGF- induced neuronal- differentiated PC12. In the future, these can also be used to study the detail mechanism of how cystatin B involves in

apoptosis.

Keywords: cystatin B, PC12, apoptosis, epilepsy.

INTRODUCTION

Cystatin B, also called stefin B, is a member of a large class of proteins that inhibit cystein proteases (Barrett et al. 1986, Turk & Bode 1991). The protein is 98 amino acid in size, distributed ubiquitously among different tissues and cell types (Turk and Bode, 1991), localized mostly intracellularly, but has been found extracellularly. Cystatin B is a tightly binding reversible inhibitor of a variety of proteases including cathepsins L, H, B, S and plant cysteine protease papain (Jarvinen and Rinne, 1982; Ritonja et al., 1985; Jerala et al., 1988). Cathepsins B, H, L, and S are localized in lysosomes, involved in general protein catabolism and possibly in specific proteolytic processing events (Mark et al., 1986; Bohely and Seglen, 1992). It has been proposed that the cystatin B serve as a protector against the proteases leaking from lysosomes, participate in the regulation of proteolysis.

This protease inhibitor was correlated to a disease when Pennacchio et al. (1996) found that mutation in cystatin B gene was associated with type I progressive myoclonic epilepsy (EPM1, also called Unverricht-Lundborg disease), one of important type of myoclonic epilepsy occurring in childhood and young adult age. They identified two cystatin B gene point mutations in EPM1 patients (Pennacchio et al. 1996), and later many other mutations in cystatin B gene (Lalioti et al. 1997a, Bespalova et al. 1997) or promoter (Lalioti et al. 1997a, 1997b, 1998) were discovered, all of them resulted in truncated proteins or decreased cystatin B mRNA level, and EPM1 phenotype. The gene encoding cystatin B is approximately 2.7 kb in length, the mRNA is about 0.8 kb and expressed in all tissues examined including brain (Pennacchio et al. 1996). EPM1 is a rare disease characterized by onset age between 6-16 years, stimulus- sensitive myoclonus and tonic- clonic seizures (Koskiniemi 1974a; Koskiniemi et al. 1974b; Norio and Koskiniemi 1979). The patients developed progressive mental deterioration and cerebellum ataxia (Norio and Koskiniemi 1979), and computed tomography (CT) scanning of patients' cerebral showed a progressive cortical atrophy (Parmeggiani et al., 1997). Gene knockout mice lacking of cystatin B expression developed profound neurological symptoms including progressive ataxia and myoclonic epilepsy, and increased apoptosis characterized by condensed nuclei and fragmented DNA was observed in in these cerebellar granule cells animals (Pennacchio et al. 1998). Recent study showed that seizure activity induced widespread expression of cystatin B mRNA and protein in rat forebrain neuron, and it was proposed that the upregulation of CSTB following seizures may counteract apoptosis (D'Amato et al., 2000). Moreover, N-acetylcysteine, a sulfhydryl antioxidant which has been shown to protect many cells from a variety of apoptotic stimuli, can effectively treat the EPM1 patients (Hurd et al., 1996; Selwa, 1999; Ben-Menachem et al., 2000).

Taking together, it is quite clear that cystatin B plays important role in the survival of neuronal cells, and lacking of cystatin B activity may hamper the well-being of central nerve system. The gene knockout mice experiment suggested that cystatin B could inhibit the apoptotic pathway in cerebellar granule cells (Pennacchio et al. 1998), however, the mechanism by which cystatin B is involved in the pathway of apoptosis is still unclear. It is also not clear why deficiency of cystatin B protein seems to attack only neural system. Other studies showed that cathepsin B and D were involved in the apoptosis of serum deprived PC12 cells (Ohsawa et al., 1998; Shibata et al., 1998; Isahara et al., 1999). Cathepsin B and L were also implicated in ischemia-caused apoptosis of gerbil and monkey hippocampus CA 1 pyramidal neuron

(Nitatori et al, 1995; Kohda et al., 1996). Cathepsin B was shown to participate in the processing of several procaspases to active caspases, which are important elements in the apoptosis (Vancompernolle et al., 1998), and the cathepsin B activities in processing of caspase zymogens and induction of nuclear apoptosis are inhibited by the synthetic peptide caspase inhibitors. Caspase-3 deficient mice die prematurely, with increased number of cerebellar granule cells due to insufficient apoptosis during development (Kuida et al. 1996), and the caspase-3 is required for the apoptosis occurs in the cultured cerebellar granule neurons (Du et al. 1997). Since cystatin B is a natural inhibitor for cathepsin B, it is possible that reduction in the expression of cystatin B might increase apoptosis by inappropriate activation of cathepsins and thereby increase the activation of caspases. Alternatively, it might increase the general proteolysis, which damages cells and lead to apoptosis of unhealthy cells.

To further explore the function of cystatin B in apoptosis, we propose to study the effect of changing in cystatin B expression on apoptosis of the PC12 cell, and to measure the apoptotic gene expression and enzyme activitie of proteases during the course of apoptosis. PC12 cell line is derived from the rat pheochromocytoma (Greene and Tischler, 1976), it is widely used because of its ability to differentiate into neuronal cells in response to nerve growth factor (NGF). Using this established cell line, it will be easier to manipulate the expression of cystatin B gene and to examine the cellular and biochemical changes during the course of apoptosis than the animal model.

RESULTS AND DISCUSSIONS:

To clarify the role of cystatin B protein in the apoptosis of neuronal cells, we cloned the cDNA of cystatin B gene in either sense or antisense orientation into the pCDNA3 expression vector. These plasmids, expressing either sense of antisese RNA of cystatin B gene, were separately transfected into the rat adrenal pheochromocytoma PC12 cells, and stable transfected cell clones were picked. The levels of cystatin B protein in these cell clones were then evaluated using immunoblotting, and the clones expressing higher level of cystatin B protein (sense cystatin B RNA) or lower level of cystatin B protein (antisense cystatin B RNA) were selected for our experiments. According to the immunoblotting results, we have obtained several independent clones of cystatin-B overexpressing and underexpressing PC12 clones. The high/low cystatin B cell clones were subjected to various conditions, including high concentration of arachidonic acid, serum deprivation, hydrogen peroxide, and the toxicity were evaluated by LDH assay. This will allow us to understand which pathway of apoptosis can be inhibited by the cystatin B protein. In the coming future, we will induce these PC12 cell clones to neuronal differentiation, and examine the effect of various cystatin B protein levels on the apoptosis of these differentiated cells. We also plan to study the expression of bcl-2, bax and p53 genes, and activities of cathepsin B, D, L and caspase-3 during the course of apoptosis in these NGF-induced PC12 cells, and their correlation with the cystatin B protein level.

REFERNCES:

Barrett AJ (1986) The cystatins: a diverse superfamily of cysteine peptidase inhibitors. Biomed Biochim Acta 45:1363-74

Ben-Menachem E, Kyllerman M, Marklund S (2000) Superoxide dismutase and glutathione peroxidase function in progressive myoclonus epilepsies. Epilepsy Research 40:33-39.

Bespalova IN, Adkins S, Pranzatelli M, Burmeister M (1997) Novel cystatin B mutation and diagnostic PCR assay in an Unverricht- Lundborg progressive myoclonus epilepsy patient. Am J Med Genet 74:467-71

D'Amato E, Kokaia Z, Nanobashvili A, Reeben M, Lehesjoki AE, Saarma M, Lindvall O (2000) Seizures induce widespread upregulation of cystatin B,

the gene mutated in progressive myoclonus epilepsy, in rat forebrain neurons. Eur J Neurosci 12:1687-1695.

Du Y, Bales KR, Dodel RC, Hamilton-Byrd E, Horn JW, Czilli DL, Simmons LK, et al (1997) Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. Proc Natl Acad Sci U S A 94:11657-62

Greene LA. Tischler AS. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A. 73(7):2424-8.

Hurd RW, Wilder BJ, Helveston WR, Uthman BM (1996) Treatment of four siblings with progressive myoclonus epilepsy of the Unverricht-Lundborg type with N-acetylcysteine. Neurology 47:1264-1268.

Isahara, K., Ohsawa, Y., Kanamori, S., Shibata, M., Waguri, S., Sato, N., Gotow, T., Watanabe, T., Momoi, T., Urase, K., Kominami, E., and Uchiyama, Y. (1999). Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. Neuroscience *91*, 233-49.

Jarvinen M and Rinne A. Human spleen cysteineproteinase inhibitor. Purification, fractionation into isoelectric variants and some properties of the variants. Biochim. Biophys. Acta 708, 210 (1982)

Jerala R, Trstenjak M, Lenarcic B, and Turk B. Cloning a synthetic gene for human stefin B and its expression in E. coli. FEBS Lett. 239, 41 (1988).

Kohda, Y., Yamashima, T., Sakuda, K., Yamashita, J., Ueno, T., Kominami, E., and Yoshioka, T. (1996). Dynamic changes of cathepsins B and L expression in the monkey hippocampus after transient ischemia. Biochem Biophys Res Commun *228*, 616-22.

Koskiniemi M, Donner M, Majuri H, Haltia M, Norio R (1974a) Progressive myoclonus epilepsy. A clinical and histopathological study. Acta Neurol Scand 50:307-32

Koskiniemi M, Toivakka E, Donner M (1974b) Progressive myoclonus epilepsy. Electroencephalographical findings. Acta Neurol Scand 50:333-59

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, et al (1996) Decreased apoptosis in the brain and premature lethality in CPP32- deficient mice. Nature 384:368-72

Lalioti MD, Mirotsou M, Buresi C, Peitsch MC, Rossier C, Ouazzani R, Baldy-Moulinier M, et al (1997a) Identification of mutations in cystatin B, the gene responsible for the Unverricht-Lundborg type of progressive myoclonus epilepsy (EPM1). Am J Hum Genet 60:342-51

Lalioti MD, Scott HS, Buresi C, Rossier C, Bottani A, Morris MA, Malafosse A, et al (1997b) Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy [see comments]. Nature 386:847-51

Lalioti MD, Scott HS, Genton P, Grid D, Ouazzani R, M'Rabet A, Ibrahim S, et al (1998) A PCR amplification method reveals instability of the dodecamer repeat in progressive myoclonus epilepsy (EPM1) and no correlation between the size of the repeat and age at onset. Am J Hum Genet 62:842-7

Marks N. Berg MJ. Benuck M. Preferential action of rat brain cathepsin B as a peptidyl dipeptidase converting pro-opioid oligopeptides. Archives of Biochemistry & Biophysics. 249(2):489-99, 1986

Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibanai, K., Kominami, E., and Uchiyama, Y. (1995). Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci *15*, 1001-11.

Norio R, Koskiniemi M (1979) Progressive myoclonus epilepsy: genetic and nosological aspects with special reference to 107 Finnish patients. Clin

Genet 15:382-98
Ohsawa, Y., Isahara, K., Kanamori, S., Shibata, M., Kametaka, S., Gotow, T., Watanabe, T., Kominami, E., and Uchiyama, Y. (1998). An ultrastructural and immunohistochemical study of PC12 cells during apoptosis induced by serum deprivation with special reference to autophagy and lysosomal cathepsins. Arch Histol Cytol *61*, 395-403.
Parmeggiani A, Lehesjoki AE, Carelli V, Posar A, Santi A, Santucci M, Gobbi G, Pini A, Rossi PG (1997) Familial Unverricht-Lundborg disease: a clinical, neurophysiologic, and genetic study. Epilepsia 38:637-641.
Pennacchio LA, Lehesjoki AE, Stone NE, Willour VL, Virtaneva K, Miao J, D'Amato E, et al (1996) Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). Science 271:1731-4
Pennacchio LA, Bouley DM, Higgins KM, Scott MP, Noebels JL, Myers RM (1998) Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. Nat Genet 20:251-8
Ritonja A. Machleidt W. Barrett AJ. Amino acid sequence of the intracellular cysteine proteinase inhibitor cystatin B from human liver. Biochemical & Biophysical Research Communications. 131(3):1187-92, 1985
Selwa LM (1999) N-acetylcysteine therapy for Unverricht-Lundborg disease. Neurology 52:426-427.
Shibata, M., Kanamori, S., Isahara, K., Ohsawa, Y., Konishi, A., Kametaka, S., Watanabe, T., Ebisu, S., Ishido, K., Kominami, E., and Uchiyama, Y. (1998). Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation. Biochem Biophys Res Commun *251*, 199-203.
Turk V, Bode W (1991) The cystatins: protein inhibitors of cysteine proteinases. FEBS Lett 285:213-9
Vancompernolle, K., Van Herreweghe, F., Pynaert, G., Van de Craen, M., De Vos, K., Totty, N., Sterling, A., Fiers, W., Vandenabeele, P., and Grooten, J. (1998). Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. FEBS Lett *438*, 150-8.