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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※Calreticulin 在神經母細胞瘤病理成因的角色 ※
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※The Role of Calreticulin in the Pathogenesis of ※
※Neuroblastoma ※
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計畫編號：NSC　91－2314－B－002－255－
執行期間：91 年　8 月　1 日至　92 年　7 月 31 日

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中　華　民　國　92　年　8　月　27　日

行政院國家科學委員會專題研究計畫成果報告

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中文摘要

Calreticulin(CRT)是一種細胞內質網蛋白質，目前我們初步的免疫染色結果顯示 CRT 的表現會隨組織分化而逐漸增加。而有表現 CRT 的病患的整體存活率也較不表現 CRT 的病患要好。然而 CRT 在神經母細胞瘤的角色仍需加以釐清。本計畫以神經母細胞瘤細胞株為模型，在體外刺激神經母細胞瘤的細胞株分化，結果顯示 CRT 會隨神經母細胞瘤細胞分化而增加，而且 CRT 在細胞死亡時仍維持高度表現。進一步將 CRT 轉殖入經母細胞瘤細胞則會促使細胞凋亡。同時以二維電泳的方法來檢測細胞株在不同的分化時期及凋亡時之相關的蛋白質改變。結果顯示細胞株分化後，共有 39 種蛋白質的表現量增加而有 40 種減少；細胞株凋亡時則有 61 種蛋白質的表現量增加而有 22 種減少。本計畫顯示 CRT 可同時影響神經母細胞瘤細胞的分化及凋亡，並進而影響其生物行為。進一步探討 CRT 與其他在分化及凋亡時發生變化的蛋白質的關係，將可更加了解 CRT 在神經母細胞瘤病理成因的角色。

關鍵詞：Calreticulin、神經母細胞瘤、病理成因、細胞分化、細胞凋亡

Abstract:

Calreticulin(CRT) is a member of endoplasmic reticulum protein. Our immunohistochemical results show that the positive immunoreactive rate of CRT increases as the histology becomes more differentiated. Patients with positive expression of CRT have a better overall survival rate. Our preliminary results confirm that CRT plays an important role in NB, and that CRT can be a prognostic factor of NB. However, the real association between CRT and differentiation of NB remains to be clarified. To disclose the role CRT in the pathogenesis of NB, this project comprises *in vitro* studies by using NB cell lines. The protein levels of CRT increased after NB cells were induced to differentiate and remained high after the cells going to apoptosis. The NB cells go to apoptosis after the cells were transfected with CRT gene. Furthermore, two-dimensional electrophoresis showed 39 proteins increased and 40 decreased after the cells differentiated, and 61 proteins increased and 22 decreased after the cells showing apoptosis. Our results revealed that CRT plays roles both in the differentiation and apoptosis of the NB cells. Further study of the relationship between CRT and the associated protein changes after the differentiation and apotosis of the NB cells, may disclose the role of CRT involved in the differentiation and apoptosis of NB cells, and also its role in the pathogenesis of NB.

Keywords: Calreticulin, Neuroblastoma, Pathogenesis, Cell differentiation, Apoptosis

Background and Purpose:

The pathogenesis of NB remains obscure. NB may exhibit three distinct patterns of behavior: life-threatening progression; maturation to ganglioneuroblastoma(GNB) or ganglioneuroma(GN); and spontaneous regression[1]. NB cells has a great potential to differentiate into mature cells. Ijiri et al showed that 8 out of 12 cases from mass–screen, after 2-18 months of observation, were of differentiating NB, GNB or GN pathologically [2]. In vitro, many chemicals, such as retinoic acid, butyric acid and cisplatin, have been shown to be able to induce NB cells to differentiate [3]. Many normally expressed molecular markers in embryonic NB cells, such as HNK-1, neuropeptide Y, Tyrosine hydroxylase, TRK and CD44 were found in NB tumors [4,5], suggesting that the NB arises during the developmental stage of the embryonic sympathetic system. Furthermore, apoptosis-related genes have been shown to express in NB tumors and that patients with NB tumors showing more apoptosis would have a higher survival rate [6]. These lines of evidence suggest that failure of either differentiation or regression by apoptotic death of NB cells is critical for the development of clinical NB.

Calreticulin(CRT) is a member of endoplasmic reticulum protein with two major functions: (i) molecular chaperoning and (ii) regulation of Ca^{2+} homeostasis [7]. CRT can also modulate cell adhesion, integrin-dependent Ca^{2+} signaling [8]. and steroid sensitive gene expression both *in vitro* and *in vivo* [9]. CRT is also one kind of stress protein and its expression is up-regulated under some stress conditions [10]. It may affect the cell sensitivity to apoptosis and it is found to be over-expressed in highly apoptotic regions of the embryo [10]. It has been demonstrated that CRT knockout mice has brain defects [11], suggesting that CRT might play a role in the development of nervous system. CRT has been found on the surface of a NB cell line and it is essential for the neurite formation when the cells are induced to differentiate [12,13]. Johnson et al [14] in

their NB cell line study, showed that CRT protein levels increase markedly when these cells are induced to differentiate by treating them with N,N-dibutyryl cAMP. Altogether, CRT could possibly play a role in the differentiation, apoptosis and angiogenesis of NB tumors and thus CRT might play a role in the pathogenesis of NB. Our preliminary results showed that the expression of CRT increased as the histology of NB tumors became differentiated. In addition, increased expression of CRT in tumor tissue predicts a better outcome. These results suggest that CRT plays important roles in the differentiation and regression of NB, and thus may contribute to the pathogenesis of NB. The aim of this study is to clarify the role of CRT in the pathogenesis of NB.

Materials and Methods:

Cell culture and treatment: Human NB cell line IMR-32 and mouse NB cell line Neuro-2A (ATCC No. CCL-131) are cultured in 90% MEM (Eagle) supplied with non-essential amino acids and Earle's BSS and 10% fetal bovine serum and penicillin-streptomycin in a 5 % CO_2 humidified incubator at 37ºC. For treatment, $1x10^5$ cells are seeded in each 6 mm well of a 6-well plate, then 25 μ M All-trans retinoic acid (ATRA) or c-AMP(Sigma, St. Louis, Missouri) dissolved in DMSO are added in each well to induce differentiation [15].

Detection of cell differentiation and expression of CRT: Morphological differentiation is measured from photographs of the cultures after treated with ATRA or c-AMP for 3, 4, 5, 6, 7 and 8 days and percentage of cell differentiation characterized by cells with processes longer than 50 μ m[15] are recorded. From each day as above, treated and untreated cells are harvested with trypsin and washed with PBS and lysed with EDTA. Proteins are separated by SDS-PAGE and transferred to nitrocellulose membrane. The presence and amount of NSE and CRT are determined by standard immunoblotting procedures with mouse anti-NSE antibody (Dako) and rabbit anti-CRT antibody (Upstate Biotechonology Inc. N.Y. USA). Cell differentiation is detected by increased expression of NSE [15].

Plasmids and transfection: pCMV-hCRT contained full-length human CRT DNA sequence is a kind gift from Professor Shuang-En Chuang (National Health Institute, ROC) The CRT cDNA sequence is cleaved from the pCMV-hCRT with EcorI and subcloned into pTracer-CMV2 (Invitrogene) which contains green fluorescent protein (GFP) reporter gene. This new vector is named pGFP-hCRT. The cDNA is confirmed by Northern blot. The liposome method is used for transfection of the NB cells. $2.5x10^5$ cells are seeded in the 6 mm well and are ready to be transfected until cell density reaching 50-60%. Four μg pGFP-hCRT is vigorously mixed with 12 μl TransFast™ and apply on cells for 1 hour. The success of transfection is visualized by the green color of GFP in the cell and also detected by over-expressed CRT in Western blotting. Cells transfected with pTracer-CMV2 serves as control. After transfection for 24 and 48 hours, the cell numbers in pGFP-hCRT group and control group are checked by direct counting GFP positive cells in 100x fluorescent microscope field. Ten fields are counted and numbers are averaged.

Two-dimensional Electrophoresis: NB cells in different days after treating with differentiating agents are solubilized in 200 μ l lysis buffer containing 7 M urea (Bio-Rad), 2 M thiourea, 4% CHAPS, 2% DTT, and 2% IPG buffer(pH 4-7). Aliquots containing $\sim 5x10^6$ cells are applied onto IPG(Immobilized PH Gradients) strips which have been rehydrated with lysis buffer. Isoelectric focusing using IPGphor™(Amersham Pharmacia Biotech, UK) is then conducted using pH 4-7 carrier ampholytes at 700V for 16 hours, followed by 1000V for an additional 2 hours. The first-dimensional gel is loaded onto the second-dimensional gel, after equilibration in 125 mM Tris, pH6.8, 10% glycerol, 2% SDS, 1% dithiothreitol, and bromphenol blue. For the second-dimensional separation, a gradient of 11-14% of acrylamide (Serva, Crescent Chemical, Hauppauge, NY) is used. Proteins are visualized by silver staining of the gels.

Computerized Quantitative Analysis: Analysis of the protein profiles obtained by 2-D electrophoresis is performed by densitometric scanning of the silver-stained gels, digitalization of the image, computer-assisted comparison of protein profiles, quantitation of protein differences, and determination of the molecular masses and pI values of the proteins of interest. These procedures are implemented with a Sun Microsystems 3/110 computer interfaced to a Photometrics scanner and digitizer (PS200 power supply, 1035x1320 assay). The gels are scanned with a Nikon f2.8 macro lens, and the output is digitized in such a manner that the background noise is subtracted from the protein spot signal. The data are stored on the main computer, and comparative analysis of the protein profiles is performed with ELSIE-5 software program.

In-gel Enzymatic Digestion: The proteins of interest are excised from the two-dimensional gels and destained for 5 minutes in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. Following washes with water, the gel pieces are dehydrated in 100% acetonitrile for 5 minutes and dried in a vacuum centrifuge. Digestion is performed by addition of 100 ng of trypsin in 200 mM ammonium bicarbonate. The Lys-C digestion is

performed with 500 ng of the endoproteinase Lys-C (Roche Molecular Biochemicals) in 100 mM Tris-HCl, pH 9. Following enzymatic digestion overnight, the peptides are extracted with 50 μl of 60% acetonitrile, 1% trifluoro-acetic acid. After removal of acetonitrile by centrifugation in a vacuum centrifuge, the peptides are concentrated for further analysis.

Statistical analysis: The statistical analyses are performed with SPSS 10.0 for window software. Comparison of two sample means is assessed by t-test.

Results:

CRT Expression and Cell Differentiation *In Vitro*

Neuro-2A cells showed morphologic differentiation with extensive neurite outgrowth after treatment with RA for 3 days and the percentage of differentiating cells increased during treatment for 7 days, after which the differentiated cells began to withdraw neurites and showed condensing of the cytoplasm and nucleus, and eventual detachment and death of rounded cells (Fig. 1, A). NSE immunoblotting confirmed that the expression of NSE increased on day 3 of RA treatment and reached a plateau on day 5, then began to decrease beginning on day 7 (Fig. 1, B). CRT expression also showed a linear increase with the differentiation of the NB cells (Fig. 1, B), however, in contrast to NSE, the expression of CRT remained high after RA treatment for 7 days when the cells began to die. The results for IMR-32 cells treated with either c-AMP or RA and Neuro-2A cells treated with c-AMP were similar to the results of Neuro-2A treated with RA (data not shown).

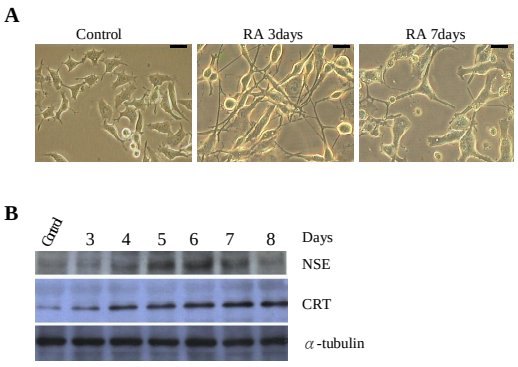


Fig. 1. Induction of differentiation and death in Neuro-2A cells by RA. A, morphologic changes after treatment with RA for 3 days (RA 3 days) and 7 days (RA 7 days), scale bars = 20μm. B, protein changes of NSE and CRT after treatment with RA for 3 to 8 days, α-tubulin served as internal control.

Effect of CRT Transfection on NB Cells

The success of transfection was visualized by the green color of green fluorescent protein in the cells on fluorescence microscopy (Fig. 2, A), and also shown by the over-expression of CRT in immunoblotting (Fig. 2, B). NB cells transfected with CRT gene died rapidly without obvious morphologic differentiation. Statistical data showed a significant decrease in cell numbers by 20% after transfection of CRT gene for 24 hours and by 50% after 48 hours as compared with the vector group (Fig. 2, C). Before transfection, there was no difference in the percentage of apoptotic cells in both the pGFP-hCRT group and the vector group (1.8 ± 0.4% and 1.6 ± 0.3% respectively). However, after transfection for 24 hours, apoptosis was observed in 13.4 ± 0.8% of cells in the pGFP-hCRT group and only in 5.1 ± 0.5% of cells in the vector group (P = 0.0003, t test).

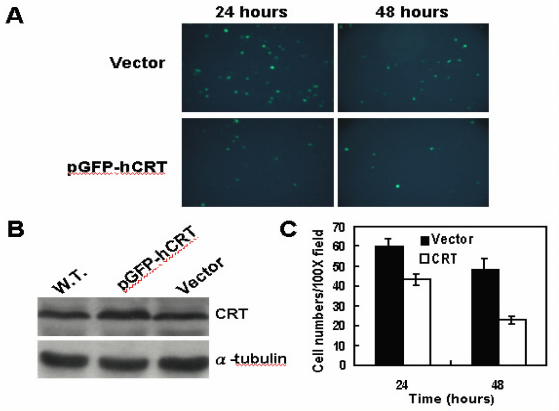
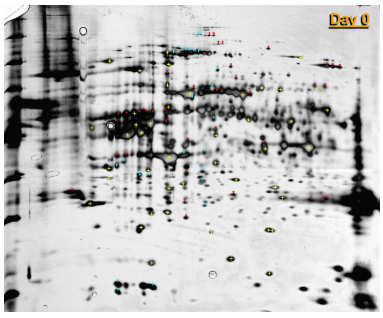


Fig. 2. Effect of CRT transfection on NB cells. A, fluorescent microscopic pictures show Neuro-2A cells after transfection with pTracer-CMV2 and pGFP-hCRT for 24 and 48 hours. B, over-expression of CRT in Neuro-2A cells transfected with pGFP-hCRT. C, Neuro-2A cells transfected with pGFP-hCRT show significantly decreased viable cells after 24 and 48 hours, error bars represent 95% confidence intervals.

Two-Dimensional Electrophoresis

The 2-D electrophpresis analysis of NB cells untreated and treated with RA for 5 and 7 days are shown in Fig. 3. There are 39 proteins increased and 40 decreased after the cells differentiate, and there are 61 proteins increased and 22 decreased after the cells go to apoptosis.



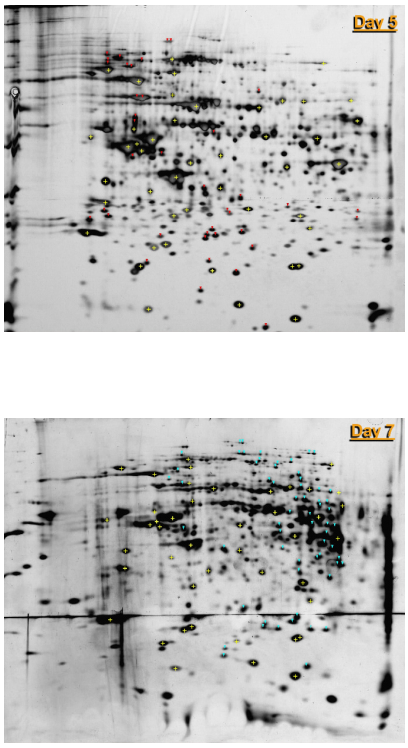


Fig. 3. 2-D electrophoresis analysis of Neuro-2A cells untreated (Day 0) and treated with RA for 5 (Day 5) and 7 (Day 7) days. "+" indicates the landmark spots. The "arrow heads" (small colored triangles) are the spots that are differentially present. Red ones are the differences between Day 0 and Day 5. Blue ones are those between Day 0 and Day 7.

Four proteins decreased after cell differentiation were identified as Cyclophilin-A, Thioredoxine peroxidase, Ribosomal protein S12, and Macrophage migration inhibitory factor. Four proteins increased after cell differentiation were also identified as GRP78, Ferritin light chain 1, Biphosphatase neuleotidase 1, and Tropomyosin.

Discussion:

In concert with our *in vivo* results, our *in vitro* studies showed that the protein levels of CRT increased gradually as the NB cells differentiated. It was also interesting to find the concurrence of high CRT levels and death of the NB cells, suggesting that CRT might also have been involved in the death of NB cells. Our study further showed that over-expression of CRT by transfection could rapidly lead to death of NB cells by inducing apoptosis, a possible mechanism of spontaneous regression of NB [16-18]. In fact, as shown by our *in vivo* studies, although there was a positive correlation between CRT expression and histologic grade of differentiation, only CRT expression but not histologic grade of differentiation had prognostic value. This result supports the notion that CRT negatively regulates the growth of NB cells by affecting more than cell differentiation alone. Altogether, the concordance of *in vivo* and *in vitro* studies strongly suggest that increased CRT expression likely contributes to a favorable prognosis in patients with NB by concomitantly affecting differentiation and apoptotic death of NB cells.

The exact role of CRT in the differentiation of NB cells remains unknown. Xiao et al suggested that cell surface CRT could regulate neurite formation via interaction with extracellular matrix proteins via its lectin-binding site [12]. In addition, surface CRT can also form a bi-directional signaling complex with the integrin α subunit that is essential for cell adhesion, spreading and differentiation [12]. However, as only a small percentage of CRT (about 5%) is found on the cell surface and this percentage being similar between differentiated and undifferentiated cells, the increased CRT levels in differentiated NB cells most probably played roles other than on the cell surface [13]. On the other hand, NB cells expressing Trk-A, a NGF receptor, may display differentiation in the presence of NGF [19]. Trk-A, after activation by NGF, may promote intracellular signaling cascades, including the Ras/ERK protein kinase pathway, the PI3K/Akt kinase pathway, and PLC-γ1 [20]. Activated PLC-γ1 acts to hydrolyze phosphatidylinositides to generate diacylglycerol, which may further activate the PKC-δ [20]. PKC-δ in turn is required for activation of the ERK cascade and for neurite outgrowth [20]. Interestingly, it has been shown that CRT is a substrate and binding protein for all PKC isoforms, and behaves like a RACK protein, suggesting that CRT plays an important role in the common PKC activated signaling pathway [21]. Thus, it is conceivable that CRT may participate in the process of Trk-A mediated NB cell differentiation. In our transient transfection study, most NB cells after transfection with CRT gene died out within 3 days. In this period, we could not observe any morphologic or biologic changes of differentiation. Three days were probably too short to study the phenomenon of differentiation, as we have shown that most NB cells exhibited obvious differentiation only after treatment with RA for 3 days. To further investigate the role of CRT in the differentiation of NB cells, studies to establish a CRT-inducible clone of NB cell line are needed.

So far we have identified 8 proteins changes after the differentiation of the NB cells. Yet the association of these proteins and CRT remained unknown. GRP78 is another molecular chaperone in the ER, and shares similar functions as CRT. This finding suggests that molecular chaperones in the ER may all have impacts on the differentiation and apoptosis of NB cells. We will study the role of GRP78 in NB first in the future. In addition, we will also further work on identifying other proteins changing after NB cells differentiation and apoptosis.

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