行政院國家科學委員會專題研究計畫 成果報告

探討人類微小病毒 B19 之非結構蛋白 NS1 誘發細胞凋亡與自體免疫疾病之關係的研究
研究成果報告(精簡版)

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Introduction

Human parvovirus B19 (B19) was discovered in 1975 [1] and has been associated with a variety of clinical manifestations [2]. It is the causative agent in erythema infectiosum (EI). Additionally, B19 infection is associated with autoantibodies [3-5] and autoimmune diseases [5-8]. However, the mechanism by which these B19-associated diseases affect the patient is poorly understood. B19 is a small single-stranded DNA virus that contains 5596 nucleotides, and it has two large open reading frames [1]. The genes on the left side of the genome encode the nonstructural protein (NS1) and those on the right side encode two capsid proteins (VP1 and VP2). The cytotoxicity of NS1 to erythroid cells has been reported and is related to the pathogenesis of B19 virus infection [9-10]. NS1 has been reported to function as a transcription regulator by directly binding with the DNA sequence of the p6 promoter and with the Sp1/Sp3 transcription factors [9-10]. NS1 has also been shown to be involved in DNA replication, cell cycle arrest and the initiation of apoptosis [11-12].

In our previous studies, the B19 NS1 gene has been constructed into a cytomegalovirus episomal vector, pEGFP-C1 and transfected into epithelial cells COS-7. Our experimental results suggest that the cell death of the NS1-transfected cells is associated with the mitochondria related apoptosis [13-14]. Additionally, the NS1 gene mutated at amino acid position of 334 was also constructed into pEGFP-C1 and transfected into COS-7 and no apoptosis was observed. This result indicated that the NS1K334E mutant lost its ability to induce apoptosis. Apoptosis is a physiological process of cell death required to ensure that the rate of cell division is balanced by the rate of cell death in multicellular organisms. Many characteristics of the mechanisms leading to apoptosis in cells in various activations states or through different stimuli engaging several intracellular signaling cascades have been described in the last years. Dysregulation of apoptosis is associated with the pathogenesis of a wide array of diseases: cancer, neurodegeneration, autoimmunity, heart disease and others [15]. However, the molecular mechanism of B19 NS1-mediated apoptosis and autoimmunity has not been clarified. Present study addresses the issue whether cellular antigens recognized by antinuclear autoantibodies are driven by apoptosis from B19 infection.

Materials and methods

Cell culture and Transfection

COS-7 and Hela cells were originally obtained from American type culture collection (ATCC) (Manassas, Va, USA) and were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL, Carlsbad, California, USA) at 37℃ and 5% CO_2 incubator. A total of $1x10^6$ cells were grown to 70% confluent in 100 mm^2 culture plates before transfection. The transfection reaction was performed by using Lipofectamine plus reagents (Invitrogene, California, USA) with 2 μg of each plasmid, pEGFP-C1, the pEGFP-NS1 or pEGFP-NS1K334E constructant according to the manufacture's instruction. The cells were then cultured in serum-free DMEM for 12 hr at 37

℃ in a 5% CO_2 incubator and subsequently in DMEM with 10% FBS. Expressions of EGFP alone or EGFP-NS1/ENFP-NS1K334E fusion proteins were examined by using fluorescence microscopy, immunoblot analysis, and flow cytometric analysis.

Fluorescence analysis

EGFP, EGFP-NS1 and EGFP-NS1K334E expression in transfected cells were observed with a Zeiss Axioplan-2 epifluorescence microscope equipped with a fluorescence filter. Digital images of the cells were recorded by using a spot camera system. The cells (~2 x 10^6) were fixed in 75% alcohol for 12-16 hr at 4℃, followed by RNase (1 mg/mL) treatment at 25℃ for 30 min. Cells were stained with propidium iodide (PI, 10 μg/mL) for 30 min before cell cycle analysis with a flow cytometer (FACScan, Becton Dickinson, Bedford, MA, USA).

Cell extraction, SDS-PAGE and Western blot

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping in 1 ml of ice-cold PBS. Pellets were resuspended in 40 ul of PRO-PREP™ buffer (iNtRON Biotech) and incubated on ice for 30 min. The lysates were removed by centrifugation at 15,000x g for 10 min to remove the insoluble component. Samples were immediately aliquoted and stored frozen at -20 ℃. Protein concentration of tissue extracts was determined according to the method described by Bradford using bovine serum albumin as standards. Five hundred μg of protein from the supernatants were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were applied to the gel and separated at 100-150 V for 1.5 hr. The gel was then electrophoretically transferred to nitrocellulose, according to the method of Towbin et al [16]. The nitrocellulose-transferred proteins were cut into strips and soaked in 5% nonfat dry milk in PBS, for 30 min at room temperature, to saturate irrelevant protein binding sites. Antiserum diluted with 5% nonfat dry milk in PBS (1:100) were reacted with the nitrocellulose strips and incubated for 1.5 hr at room temperature. The strips were washed twice with PBS-Tween for 1hr and adding secondary antibody consisting of alkaline phosphatase conjugated goat anti-human or mouse IgG antibodies (Sigma) for 1hr at room temperature. The substrate of nitroblue tetrazolium/5-bromo-4-chloro-3 indolyl phosphate (NBT/BCIP) was used to detect antigen-antibody complexes. Anti-Sm, Anti-RNP, Anti-SS-A, Anti-SS-B, Jo-1, Anti-centromere antibody (ACA) and Scl-70 antibody were purchased from MBL (Japan). Anti-MPO was purchased from INOVA Diagnostics, Inc (San Diego, CA, USA). Anti-Ku was purchased from BD Pharmingen (USA). Anti-actin was purchased from Santa Cruz biotechnology, inc. (Santa Cruz, CA, USA).

Results and Conclusion

The plasmids pEGFP-NS1 and pEGFP-NS1K334E were constructed and transfected into COS-7 and Hela cells. The transfected cell lines were analyzed for the mechanism of apoptosis. We achieved 50-70% transfection efficiency. COS-7 and Hela cells expression EGFP-NS1 or EGFP-NS1K334E fusion proteins revealed specific cytoplasmic staining by immunofluorescence, while the cells expression EGFP only revealed both nuclear and cytoplasmic staining by immunofluorescence. In our results, we found that the mutant cells

showed decreased death in repeated experiments. The average percentages of COS-7 cells apoptosis were 18%, 6%, and 4% for cells transfected with the wild-type NS1, NS1 K334E, and the control vector, respectively. The average percentages of Hela cells apoptosis were 15%, 3%, and 2% for cells transfected with the wild-type NS1, NS1 K334E, and the control vector, respectively.

To clarify the association between NS1-transfected cells and various autoantibodies, we firstly examined the binding activities of B19-NS1 transfected cell proteins with various autoantibodies against Jo-1, SSA, SSB, Ku, ACA, MPO, Scl-70, RNP, and Sm that were obtained from commercial kits. Assay cell extracts 24 (A), 48 (B), 72h (C) after the start of transfection. Auotoantibody against Jo-1, SSA, Ku, RNP, and Sm were cross-reacted with B19-NS1 transfected cells (COS-7) (Fig 1A, B, C), but not other autoantibodies against ACA, MPO, SSB or Scl-70. Auotoantibody against Jo-1, SSA, SSB, Ku, RNP, and Sm were cross-reacted with B19-NS1 transfected cells (Hela) (Fig 2A, B, C), but not other autoantibodies against ACA, MPO or Scl-70. Increased expressions of autoantigen were found in Hela transfected cells. In additionally, there were no significantly increased expressions of autoantigen after transient transfection (Fig 1A, B, C; Fig 2A, B, C), and between mock, EGFP-, EGFP-NS1-, and EGFP-NS1K334E transfected cells were found by immunoblotting assay. These findings may provide information for further study in B19 infection and characterization of B19 NS1 protein in autoimmunity and non-erythroid linkage cells, and these will be used as reference to further study in B19 infection and pathogenesis.

計畫結果自評部分

研究內容與原計畫相符程度100%, 為探討人類微小病毒B19之非結構蛋白NS1誘發細胞凋亡與自體免疫疾病之關係的研究,達預期目標80%, 此一模式建立可以更詳盡了解NS1 protein 對 B19 感染及其非結構蛋白 NS1 對於細胞自體抗原之影響，並作為未來人類微小病毒 B19 感染及自體免疫致病機轉相關研究的參考。

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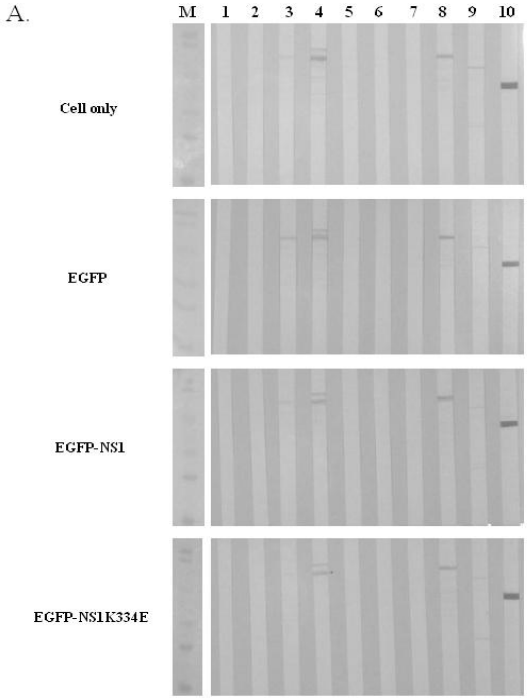

Fig 1

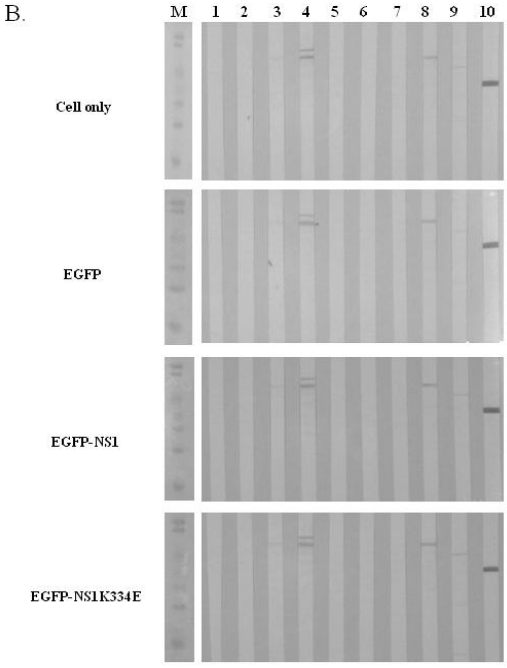

Fig 1

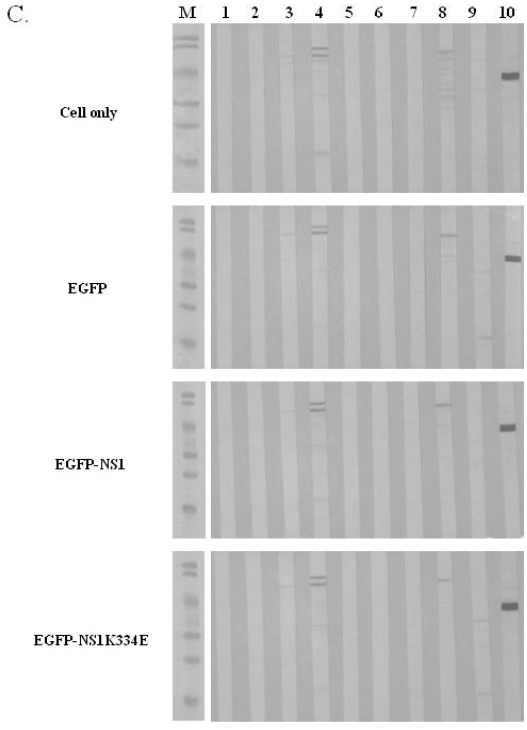

Fig 1

Fig 1. Immunoblots of mock, EGFP vector, EGFP-NS1 and EGFP-NS1K334E transfected cell of COS-7 proteins by various antibodies. Cell extracts were probed with specific autoantibodies that were purchased from the MBL (Japan). Lane 1 indicates the pre-stained marker. Lanes 2, 3, 4, 5, 6, 7, 8, and 9 represent the results of immunoblotting probed with human anti-Jo-1, anti-SSB, anti-SSA, anti-Ku, anti-centromere antibodies, anti-myeloperoxidase antibodies, anti-Scl-70, anti-RNP antibodies, and anti-Sm antibodies, respectively. Lane 10 represents the result of immunoblotting probed with actin. Assay cell extracts 24 (A), 48 (B), 72h (C) after the start of transfection. Three independent experiments were performed and similar results were observed.

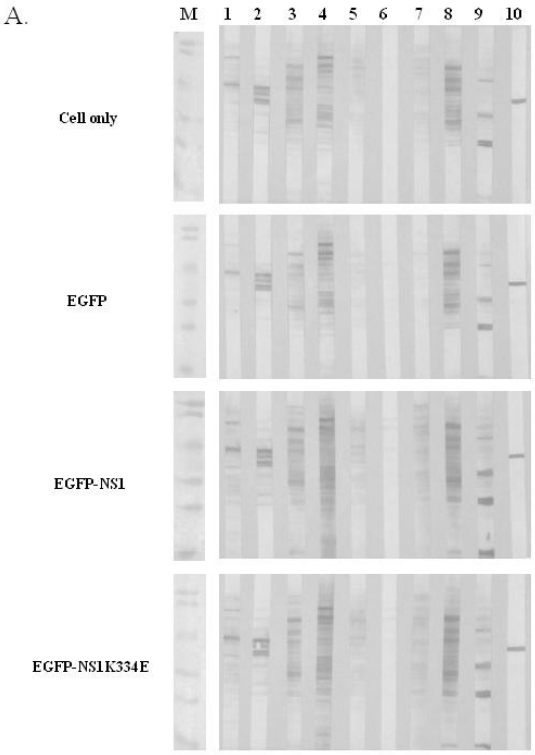

Fig 2

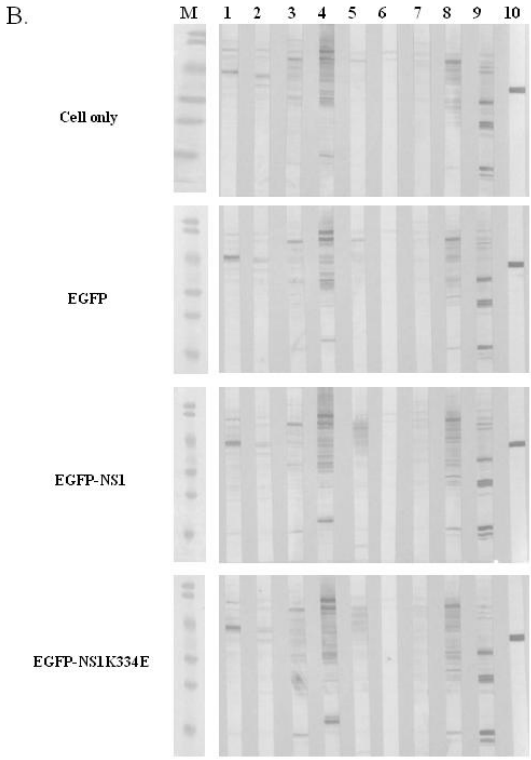

Fig 2

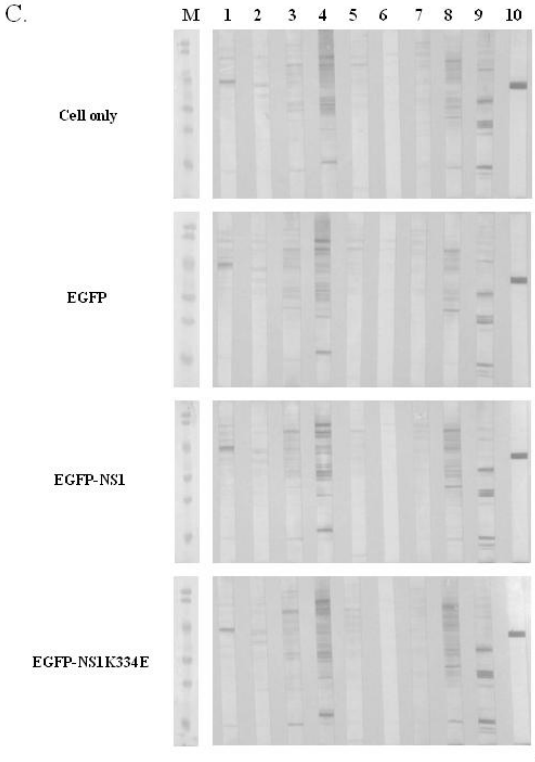

Fig 2

Fig 2. Immunoblots of mock, EGFP vector, EGFP-NS1 and EGFP-NS1K334E transfected cell of Hela proteins by various antibodies. Cell extracts were probed with specific autoantibodies that were purchased from the MBL (Japan). Lane 1 indicates the pre-stained marker. Lanes 2, 3, 4, 5, 6, 7, 8, and 9 represent the results of immunoblotting probed with human anti-Jo-1, anti-SSB, anti-SSA, anti-Ku, anti-centromere antibodies, anti-myeloperoxidase antibodies, anti-Scl-70, anti-RNP antibodies, and anti-Sm antibodies, respectively. Lane 10 represents the result of immunoblotting probed with actin. Assay cell extracts 24 (A), 48 (B), 72h (C) after the start of transfection. Three independent experiments were performed and similar results were observed.

出席國際學術會議心得報告

計畫編號	NSC-95-2314-B-040-010
計畫名稱	探討人類微小病毒 B19 之非結構蛋白 NS1 誘發細胞凋亡與自體免疫疾病之關係的研究
出國人員姓名 服務機關及職稱	徐再靜　　中山醫學大學免疫學研究所
會議時間地點	2006.10.08 ~2006.10.12 匈牙利布達佩斯
會議名稱	免疫基因體和免疫體國際會議 (BCII 2006)
發表論文題目	VP1 unique region protein associated with the production of anti-cardiolipin antibody and anti-beta2 glycoprotein I antibody in patients with acute parvovirus B19 infection

一、參加會議經過

2006 年 10 月 8 日下午抵達會議地點東歐匈牙利首都布達佩斯之布達城的會議中心(Congress center)會場，經辦理報到後取得相關會議資料，便立刻參與開幕式的正式會議，大會會長 Andras Falus 首先歡迎大家到布達佩斯，參與此次免疫基因體和免疫體國際會議(BCII 2006):International Conference of Immunogenomic and Immunomics (A joint meeting of 2nd basic and clinical Immunogenomics and 3rd Immunoinformatics (Immunomics) conference，而這個會議最主要是集合世界各地基礎與臨床的免疫學家和生物資訊學家共同為免疫學的研究進行跨領域的整合而使得免疫學的研究進展能更有效率。10 月 9 日-10 月 12 日的會議議程將分為幾個主軸做廣泛及深入的分析，包括: Oncogenomic; Genetic redirection of the lymphocyte function; systemic modeling and computational Immunology; Artificial immune system; drug development in immune diseases; Immune-Cellome; Vaccine; Immune database and tools; Immuno-gene therapy; Inflammation; Imune intracellular signaling pathways; Allergome; Neuro-Immuno Genomics; Autoimmune Immunome.各國免疫學者及與會人士均踴躍發言並且提出意見交換，會議相當熱烈討論，而在中場會議休息時間，大家仍在場外的圓桌上進行討論，把握難得的機會和世界各地的免疫學者互相交流。在應用基因體學、蛋白質體學和生物資訊學等領域之下，免疫學的研究更快速、細膩和深入主軸。本會議約有將近 5~6 百名學者參與。在 10 月

11 日的大會議程，主辦單位更細心的安排了 One-day Course—“Bioinformatics for Autoimmunity”，這個課程參與者僅限 50 名，因此我很早就先報名參加，課程相當緊湊，早上上課下午安排實際電腦操作，由於是利用電腦軟體來模擬及設計自體免疫相關實驗，因此在電腦方面專精的學者帶領及介紹下，我也領略了科技及資訊的浩瀚及在科學的應用。在壁報論文展示方面，分為兩梯次 9-10 及 11-12 日。

二、與會心得

我所發表的壁報論文是安排在 11-12 日展示，主要和自體免疫及感染有關。在這次會議上也認識了不少世界級免疫大師，包括來自歐美和澳洲等地，在相互介紹及交換名片下，也相約下次能有機會共同進行學術交流。也深覺免疫研究的分工將更精細而快速，藉注入基因體和蛋白質體學的技術，也將使得自體免疫研究的發展更快速，進而早日能發展治癒此疾病的藥物。因此此次東歐會議之行的收穫相當豐富。而這次能順利的出國進行學術交流和參與免疫學盛會，吸取新知，實在很不容易。所幸有國科會大力的支持經費與鼓勵我們新一代的研究學者出國進行學術訪問。在此特別致上最衷心的感謝。相信在不久的將來，自己的這些努力辛苦所呈現的研究成果能在學術上受到肯定，也期待日後繼續能有機會參與國外的重要學術會議。

VP1 unique region protein associated with the production of anti-cardiolipin antibody and anti-beta2 glycoprotein I antibody in patients with acute parvovirus B19 infection

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Abstract

Objectives To elucidate the association between diagnostic patterns of human parvovirus B19 (B19) and the reactivity of the anti-B19-VP1 unique region (VP1u), cardiolipin (CL) and beta2-glycoprotein I (β2GPI).

Methods Sera from 102 clinically suspected cases of B19 infection were analyzed and classified into seven groups based on the presence of B19 DNA, anti-B19 IgM and IgG by nested PCR and ELISA. Humoral responses of anti-B19-VP1u and anti-B19-VP1uD175A IgM/IgG antibodies, the anti-cardiolipin antibody (aCL) and the anti-β2GPI antibody were assessed by Western blot analysis and ELISA. Absorption experiments were also performed to determine the binding specificity of immunoglobulins to B19-VP1u, CL and β2GPI.

Results No difference in recognition of B19-VP1u and B19-VP1uD175A proteins by anti-B19-VP1u IgG and IgM antibodies was observed in serum from patients with the B19 diagnostic patterns $DNA^+/IgM^-/IgG^+$, $DNA^-/IgM^+/IgG^+$, $DNA^+/IgM^+/IgG^+$ or $DNA^-/IgM^-/IgG^+$. None of these sera exhibited detectable antibodies against dsDNA and CCP. However, sera from patients with the diagnostic pattern $DNA^+/IgM^+/IgG^+$ had a significantly high frequency (57%) for recognition of CL and β2GPI. Furthermore, adsorption experiments were performed by adding purified B19-VP1u, which partially suppressed the reactivity of anti-B19VP1u to CL and β2GPI.

Conclusions Our experimental results indicate that anti-B19-VP1u antibodies in serum from patients with the B19 diagnostic patterns $DNA^+/IgM^+/IgG^+$ have a significantly high frequency in recognition of CL and β2GPI. Acute phase of B19 infection could be an important stage in autoantibody production in B19-infected subjects and may provide a clue for clarifying the association of role of B19-VP1u in inducing autoimmune diseases.