行政院國家科學委員會專題研究計畫　期中進度報告

比較 Calreticulin 不同 domain 和腫瘤抗原的去氧核糖核酸疫苗其抗腫瘤效用和作用機轉(1/2)

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-301-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院婦產科

計畫主持人：鄭文芳
共同主持人：陳祈安
計畫參與人員：謝長堯 李建南 蘇宜寧

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 6 月 8 日

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

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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　　93　年　6　月 8　日

一、中文摘要

關鍵詞：子宮頸癌、人類乳突病毒、預防疫苗、治療疫苗、去氧核糖核酸
疫苗、病毒顆粒疫苗

子宮頸癌是台灣女性最常好發和死亡率排名第五位的惡性腫瘤。每年全世界有大約二十萬的婦女死於子宮頸癌。子宮頸癌不但治療費用高，也需要昂貴的篩檢計畫達到早期診斷，更需要經由高額的陰道鏡作為次級篩檢。目前可利用的治療方法諸如手術、放射線治療或化學治療都會在殺害癌細胞的同時，也會破壞健康的細胞。我們極需要不只降低子宮頸癌的發生率，也需要數種較好的治療模式。免疫療法能選擇性攻擊腫瘤細胞而不會破壞正常細胞。因此免疫療法看起來將提供一個令人期待地新的治療策略。我們最近研發出數種 DNA 型態的腫瘤疫苗應用在鼷鼠動物模式上。在這些 DNA 疫苗中，Calreticulin/E7 這個嵌合分子已經被發現經由 E7 特異的免疫反應和抗腫瘤血管新生兩種機制在鼷鼠動物模式上具有抗腫瘤的效力。Calreticulin 蛋白質是由三個 domains(N domain, P domain 和 C domain)所構成。因此評估 Calreticulin 哪一段的 domain 連接 E7 抗原是否也可以引發 E7 特異的免疫反應或抗腫瘤血管新生這兩種機制達到抗腫瘤的效力將是一個吸引人的問題。在第一年的研究中我們發現 NCRT/E7,PCRT/E7 及 CCRT/E7 可以產生較高的的 E7 特異的免疫細胞及較好的腫瘤保護作用。 第二年的研究將進一步比較 NCRT/E7, PCRT/E7 及 CCRT/E7 三者在產生 E7 特異的免疫反應或抗腫瘤作用的差異處。

二、英文摘要

Keywords：cervical cancer, human papillomavirus, gene therapy, immunotherapy

Cervical cancer-the most frequent neoplasm and the third mortality rate of malignancies of the women in the world. It affects half a million women each year and results in about 200,000 women dying of cervical cancer each year worldwide. Not only are the costs of treatment high, but there is also the need for expensive screening programs for early detection, and for costly secondary screening via colposcopy. Moreover, the available forms of treatment-surgery, radiation therapy, and chemotherapy are all cytoreductive treatment modalities, so in addition to killing cancerous cells, healthy cells are also destroyed in the process. Indeed, there is a need to decrease the incidence of cervical cancer and develop better forms of its treatment. We have recently developed some DNA-formated E7-specific cancer vaccines in the murine model. Among them, calreticulin (CRT) linked to a model antigen-HPV-16 E7 has been found the antitumor effects mediated by E7-specific immune responses and anti-angiogenesis in mice vaccinated with CRT/E7 DNA. Calreticulin is composed of 3 domains- N-domain, P-domain and C-domain. Which domain(s) that links with E7 can also generate anti-tumor effect via E7-specific immunologic response and/or anti-angiogenesis is needed to be further evaluated ? In the first year's research, we found that all of the NCRT/E7, PCRT/E7 and CCRT/E7 could generate higher numbers of E7-specific CD8+ T lymphocytes and higher tumor protective effects as compared to wild-type E7. So, we will focus on comparing the differences of immunologic responses and antitumor effects between NCRT/E7, PCRT/E7 and CCRT/E7 in the second year's research.

三、研究方法

Plasmid DNA Constructs and Preparation

The generation of pcDNA3-E7 has been described previously. The generation of pcDNA3-CRT has also been described previously. There is more than 90% homology between rabbit, human, mouse, and rat CRT. For the generation of pcDNA3-NCRT, DNA encoding the N-domain of CRT, NCRT was first amplified with PCR by using rabbit CRT cDNA as the template and a set of primers, 5'-CCGGTCTAGAACGCTGCTCCCTGTGCCGCT-3' and 5'-CCCGAATTCGTTGTCCGGGCCGCACGATCA-3'. The amplified product was further cloned into the Xba1/EcoR1 site of pcDNA3 (Invitrogen Corp., Carlsbad, California, USA). For the generation of pcDNA3-PCRT, DNA encoding the P-domain of CRT was first amplified with PCR using rabbit CRT cDNA as the template and a set of primers, 5'-TGCTCTAGAATGTACAAGGGTGAGTGGAAGCC-3'凶and

5’-CCGGAATTCCAGCTCGTCCTTGGCCTGGCC-3’. The amplified product was further cloned into the XbaI/EcoRI site of pcDNA3. For the generation of pcDNA3-CCRT, DNA encoding the C-domain of CRT was first amplified with PCR using rabbit CRT cDNA as the template and a set of primers,
5’-TGCTCTAGAATGTACAAGGGTGAGTGGAAGC-3’汹and
5’-CCGGAATTCCAGCTCGTCCTTGGCCTGGC-3’. The completed product was then cloned into the XbaI/EcoRI site of pcDNA3.

For the generation of pcDNA3-NCRT/E7, PCRT/E7, and CCRT/E7, E7 was first amplified with pcDNA3 as a template and a set of primers,
5’-GGGGAATTCATGGAGATACACCTA-3’汹and
5’-GGTGGATCCTTGAGAACAGATGG-3’, and then cloned it into the EcoRI/BamHI sites of pcDNA3-NCRT, PCRT, or CCRT to generate pcDNA3-CRT/E7, pcDNA3-NCRT/E7, pcDNA3-PCRT/E7, pcDNA3-CCRT/E7.

Cell Line

The production and maintenance of TC-1 cells have been described previously [Lin, 1996 #175]. In brief, HPV-16 E6, E7 and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells to generate TC-1.

DNA Vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination were performed using a helium-driven gene gun according to a protocol described previously with some modifications [Hung, 2001 #1487]. Gene gun particle-mediated DNA vaccinations were performed using a Low Pressure-accelerated Gene Gun (BioWare Technologies Co. Ltd, Taipei, Taiwan). The gold particles (Bio-Rad 1652263) were weighted and suspended in 70% ethanol. This suspension was vortexed furiously and then centrifuged to collect the particles. After washing by distilled water three times, the collected particles were resuspended in DNA solution (1 μg DNA per mg gold particles), vortexed and sonicated for a few seconds, and then added 2.5M $CaCl_2$ and 0.05 M spermidine solution with vortex. This solution was kept on ice for ten minutes and the DNA-coated gold particles were collected and washed by 100% ethanol three times. Finally, the particles were resuspended in 100% ehanol with appropriate concentration and used to make bullets. Control plasmid (no insert), E7, NCRT, NCRT/E7, PCRT/E7, CCRT/E7, or CRT/E7 DNA-coated gold particles were delivered to the shaved abdominal region of mice using a Low Pressure-accelerated Gene Gun (BioWare Technologies Co. Ltd, Taipei, Taiwan) with a 50psi discharge pressure of helium.

Intracellular Cytokine Staining and Flow Cytometry Analysis

Mice were immunized with 2 μg of the various DNA vaccines and received a booster with the same regimen 1 week later. Splenocytes were harvested 1 week after the last vaccination. Before intracellular cytokine staining, $5x10^6$ pooled splenocytes from each vaccination group were incubated for 16 hours with either 1 μg/ml of E7 peptide (aa 49-57) containing an MHC class I epitope [Feltkamp, 1993 #234] for detecting E7-specific $CD8^+$ T cell precursors or 10 μg/ml of E7 peptide (aa 30-67) containing an MHC class II epitope [Tindle, 1995 #569] for detecting E7-specific $CD4^+$ T cell precursors. Cell surface marker staining for CD8 or CD4 and intracellular cytokine staining for IFN-γ, as well as flow cytometry analysis, were performed using conditions described previously [Cheng, 2002 #1743].

In Vivo Tumor Protection Experiments

For the tumor protection experiment, C57BL/6 mice (five per group) either received no vaccination or were immunized with 2 μg/mouse of plasmid encoding no insert, NCRT, E7, NCRT/E7, PCRT/E7, or CCRT/E7 using a gene gun. One week later, mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were subcutaneously challenged with $5xl0^4$ TC-1 cells/mouse in the right leg. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60.

四、結果

Vaccination with DNA encoding NCRT, PCRT, or CCRT linked to E7 significantly enhances the E7-specific $CD8^+$ T cell response

To determine if the different domains of calreticulin when linked with the E7 DNA vaccines could enhance E7-specific T cell–mediated immune responses in mice, we performed intracellular cytokine staining with flow cytometry analysis to characterize E7-specific $CD8^+$ and $CD4^+$ T cell precursors. As shown in **Figure 1,** vaccination with NCRT/E7, PCRT/E7, or CCRT/E7 DNA generated higher frequencies of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors when compared to mice vaccinated with E7 DNA ($p<0.01$). DNA encoding no insert was used as a negative control. Our results also indicated that the fusion of NCRT to E7 was required for enhancement of $CD8^+$ T cell activity, since NCRT DNA mixed with E7 DNA did not generate enhancement of $CD8^+$ T cell activity (data not shown). Vaccination with CRT/E7 DNA generated the highest number of E7-specific $CD8^+$ T cell precursors (220.5±18.5) when compared to the NCRT/E7 (178.0±18.5), PCRT/E7 (140.0±16.0) and CCRT/E7 (128.0±10.0) ($p<0.01$). Thus, our data suggest that NCRT/E7, PCRT/E7, and CCRT/E7 DNA vaccines are capable of enhancing the E7-specific CD8+ T cell response in vaccinated mice, although not as

strongly as CRT/E7.

Vaccination with NCRT/E7, PCRT/E7, or CCRT/E7 DNA enhances tumor protection in mice challenged with an E7-expressing tumor cell line

To determine if the observed enhancement of the E7-specific $CD8^+$ T cell response translated into a significant E7-specific protective antitumor effect, we performed an *in vivo* tumor protection experiment using a previously characterized E7-expressing tumor model, TC-1. As shown in Figure 2, 100% of mice receiving PCRT/E7, CCRT/E7, or CRT/E7 DNA vaccination also remained tumor-free 60 days after TC-1 challenge. In comparison, all mice vaccinated with wild-type E7 DNA developed tumors within 14 days of challenge. This suggests that each of the three domains of calreticulin can protect vaccinated mice against a lethal challenge with E7-expressing tumor cells when linked to the E7 antigen in a DNA vaccine.

附圖說明

Figure 1. Immunological profile of vaccinated mice using intracellular cytokine staining and flow cytometry analysis. Mice were vaccinated with DNA encoding no insert, E7, NCRT, NCRT/E7, CCRT/E7, PCRT/E7, or CRT/E7. Splenocytes from vaccinated mice were harvested 7 days after vaccination, cultured *in vitro* with MHC class I-restricted (aa 49-57) E7 peptide overnight, and stained for intracellular IFN-γ and CD8. (A) Bar graph depicting the number of antigen specific IFN-γ-secreting $CD8^+$ T cell precursors/3 x 10^5 splenocytes (mean±SD). *Note:* NCRT/E7, PCRT/E7, CCRT/E& and CRT/E7 DNA vaccines generated significantly higher E7-specific antibody responses when compared with mice vaccinated with the other DNA vaccines ($P<0.01$, one-way ANOVA).

Figure 2. *In vivo* tumor protection experiments in mice vaccinated with various DNA vaccines. Mice were immunized with DNA vaccines encoding E7, NCRT/E7, PCRT/E7, CCRT/E7 or CRT/E7 and then were challenged with TC-1 tumor cells. *Note:* 100% of mice receiving NCRT/E7, PCRT/E7 CCRT/E7, or CRT/E7 remained tumor-free 60 days after TC-1 challenge.

Figure 1

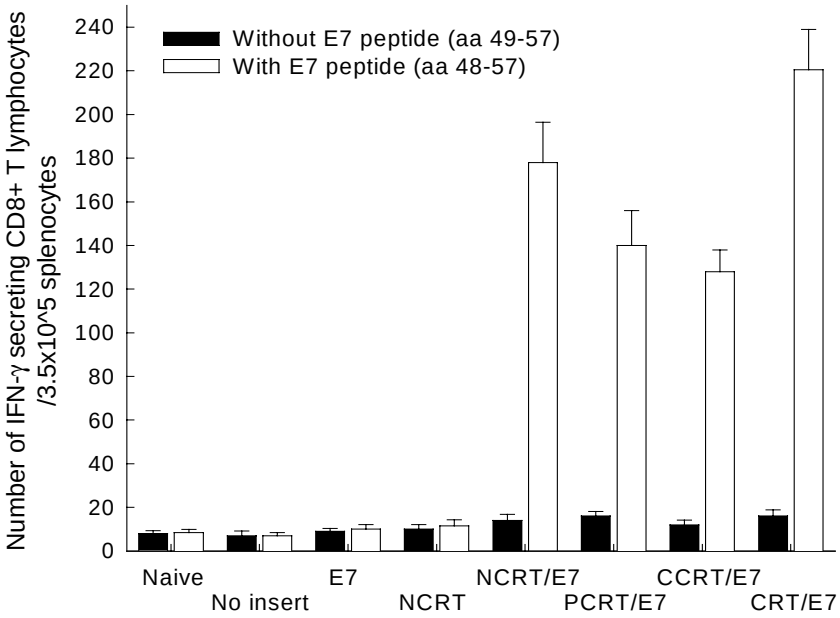

Figure 2