行政院國家科學委員會專題研究計畫　成果報告

特異抗原的 RNA 疫苗對 MHC class I 減退的腫瘤的抗腫瘤效果

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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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執行單位：國立臺灣大學醫學院婦產科
中　華　民　國　　年　　月　　日

一、中文摘要

關鍵詞：子宮頸癌、人類乳突病毒、基因療法、免疫療法

癌症免疫治療所面臨的一個主要項目是很多人類的癌症，會降低第一型主要相容複合物分子的表達。了解對抗第一型主要組織相容複合物表達下降腫瘤的抗癌效果的作用機轉將會加速合理的疫苗免疫設計和免疫治療的策略來控制這些腫瘤。SINrep5 自我複製載體載入第一型單純疱疹的 VP22 鍵結人類乳突病毒第十六型的 E7 時，可以產生對 TC-1 和 TC-1p3(A15)(第一型主要組織相容複合物下降)兩種腫瘤產生顯著地抗癌效果。對接受沒有載入任何基因或 E7 基因的 SINrep5 疫苗不能對 TC-1 但會對 TC-1p3(A15)產生保護性抗癌效果。
接受這些 SINrep5 RNA 疫苗的鼷鼠和未接受疫苗的鼷鼠產生較高百分比的自然殺手細胞。體內抗體去除，實驗顯示自然殺手細胞在 RNA 疫苗的抗 TC-1p3(A15)腫瘤效果上是很重要的。SINrep5-VP22/E7 RNA 疫苗可以控制表達第一型主要組織相容複合物和 E7 腫瘤細胞(TC-1)和第一型主要組織相容複合物下降的腫瘤細胞(TC-1p3(A15))經由不同的作用機轉。

二、英文摘要

One of the major issues facing cancer immunotherapy is that many human cancers downregulate expression of MHC class I molecules. The understanding of the mechanisms of the antitumor effects against tumors with downregulated MHC class I expression will facilitate rational design of vaccines and immunotherapeutic strategies to control such tumors. Naked Sindbis RNA replicon vector (SINrep5) encoding the herpes simplex virus type 1 (HSV-1) protein VP22 linked to E7 (SINrep5-VP22/E7) could generate significant antitumor effects against TC-1 and TC-1P3 (A15) (down-regulated MHC class I expression) tumors in vaccinated mice. Vaccination with naked SINrep5 RNA without insert or E7 vaccine could also generate protective antitumor effects against TC-1P3 (A15) but not TC-1. Mice vaccinated with any of these naked RNA vaccines generated higher percentages of NK cells compared to unvaccinated naïve mice. *In vivo* antibody depletion experiments revealed that NK cells were important for the antitumor effect generated by naked RNA vaccines against TC-1P3 (A15). SINrep5-VP22/E7 naked RNA vaccine is capable of controlling of MHC class I positive, E7-expressing tumor cells (TC-1) and MHC I-down-regulated tumor cells (TC-1P3(A15)) via different mechanisms.

三、計畫原由與目的

Self-replicating RNA vaccines (RNA replicons) are recently developed and important form of vaccines for cancer immunotherapy [1]. RNA replicon vaccines may be derived from alphavirus vectors, such as Sindbis virus [2-4]], Semliki Forest virus [5]], or Venezuelan equine encephalitis virus vector [6-8]. These vaccines are self-replicating and self-limiting and may be administered as either RNA or DNA,

which is then transcribed into RNA replicons in transfected cells or *in vivo* [9, 10]. Self-replicating RNA eventually causes lysis of transfected cells [1]]. These vectors therefore do not raise the concern of integration into the host genome associated with naked DNA vaccines. This is particularly important for development of vaccines targeting proteins that are potentially oncogenic, such as the human papillomavirus (HPV) E7 proteins.

One limitation on the potency of RNA replicon vaccines is their inability to spread *in vivo*. We have previously developed a strategy to enhance the spread of antigen by using the herpes simplex virus type 1 (HSV-1) protein VP22, which has demonstrated the remarkable property of intercellular transport and is capable of distributing protein to many surrounding cells [11]. We investigated the use of VP22 linked to a model antigen (HPV-16 E7) in the context of a Sindbis virus-derived RNA replicon vaccine (SINrep5) and explored whether it led to enhancement of antigen-specific immune responses and antitumor effects [12]. Our data indicated that a Sindbis virus RNA vaccine encoding HSV-1 VP22 linked to E7 significantly increased activation of E7-specific CD8+ T cells, resulting in potent antitumor immunity against E7-expressing tumors with normal MHC I expression [12].

One concern about the efficacy of immunotherapy is that tumors can evade immune responses through various mechanisms, including MHC class I downregulation [13]. A number of human cancers have been shown to downregulate MHC class I expression, including melanoma [14], prostate cancer [15], breast cancer [16, 17], ovarian cancer [18], and cervical cancer [19]. Therefore, it is important to assess if the self-replicating RNA vaccine can also control these tumors with lost or down-regulated MHC class I expression.

We have previously demonstrated that Vac-Sig/E7/LAMP-1, a recombinant vaccinia vaccine encoding a signal peptide linked to human papillomavirus type 16 (HPV-16) E7 and the transmembrane and the cytoplasmic domains of the lysosome associated membrane protein 1 (LAMP-1), enhances MHC class II presentation [20], improves $CD4^+$ and $CD8^+$ T cell activity [21], and generates an antitumor effect against E7-expressing tumors (TC-1) in vaccinated mice [22]. However, approximately 20% of mice treated with Vac-Sig/E7/LAMP-1 eventually develop tumors [22]. We took advantage of this breakthrough tumor to create the TC-1 P3 tumor cell line. We subjected TC-1, an MHC class I-positive E7-expressing tumor model, to three cycles of immunoselection with Vac-Sig/E7/LAMP-1. As a result, TC-1 P3 cells resisted immunotherapy by Vac-Sig/E7/LAMP-1 and demonstrated downregulation of MHC class I expression. Thus, TC-1 P3 represents a suitable tumor model for developing vaccines and immunotherapeutic strategies to control tumors with downregulated MHC class I expression.

In this study, we tested the potency of the self-replicating SINrep5 RNA vaccines by challenging mice with an HPV-16 E7-expressing MHC I down-regulated tumor cell line, TC-1 P3 (A15), which is a subclone of TC-1 P3 [23]. Our data indicated that vaccination with Sindbis virus RNA replicon vaccine (SINrep5) with no insert, or encoding wild-type E7, VP22 alone, or VP22/E7 significantly inhibited the growth of TC-1 P3 (A15). We also found that NK cells play the most important role in the anti-tumor effects mediated by the naked RNA vaccines.

四、實驗方法

Cell Lines

The production and maintenance of TC-1 cells has been described previously [22]. In briefly, HPV-16 E6, E7 and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells. This tumorigenic cell line was named TC-1. The generation and characteristics of the MHC I down-regulated tumor cell line, TC-1 P3, and its subclone, TC-1 P3 (A15), have been described previously [23]. TC-1 and TC-1 P3 (A15) tumor cells were grown in RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, 50 units/ml penicillin/ streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 2mM nonessential amino acids and 0.4 mg/ml G418 at 37^{o}C with 5% CO_2. On the day of tumor challenge, tumor cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS) and finally resuspended in 1X HBSS to the designated concentration for injection.

Mice

Six- to eight-week-old female C57BL/6J mice were purchased from the National Taiwan University (Taipei, Taiwan) and bred in the animal facility of the National Taiwan University Hospital (Taipei, Taiwan). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Plasmid DNA Constructs and *In vitro* RNA Preparation

The Sindbis virus RNA replicon vector SINrep5 has been described previously [24]. SINrep5 was kindly provided by Charles M. Rice at the Washington University School of Medicine, St. Louis, Mo.

The generation and characterization of RNA transcripts from SINrep5-VP22, SINrep5-E7, SINrep5-VP22/E7, and SINrep5 was described previously [25]. RNA vaccines were transcribed in vitro and capped using SP6 RNA polymerase and capping analog from the in vitro transcription kit (Life Technologies, Rockville, MD) according to the vendor's manual . After synthesis, DNA was removed by digestion with DNase I. Synthesized RNA was then purified by precipitation. RNA concentration was determined by optical density measured at 260 nm. The integrity and quantity of RNA transcripts were further checked using denaturing gel

electrophoresis [12]. The purified RNA was divided into aliquots to be used for vaccination in animals.

***In vivo* Tumor Protection Experiments**

For the tumor protection experiment, Six- to eight-week-old female C57BL/6J mice (five per group) were immunized intramuscularly with 1 μg/mouse of SINrep5-VP22, SINrep5-E7, SINrep5-VP22/E7, or SINrep5 (no insert) RNA vaccine in the right hind leg. Fourteen days after immunization, mice were injected intravenously with $1x10^4$ TC-1 or P3 (A15) tumor cells per mouse through the tail vein. Three or four weeks later, mice were euthanized. The lung weight and number of pulmonary nodules in each mouse were evaluated and counted by experimenters in a blinded fashion.

***In vivo* Antibody Depletion Experiments**

The procedure for in vivo antibody depletion has been described previously [4]. In brief, mice (five per group) were vaccinated with 1 μg of self-replicating SINrep5 with or insert or VP22/E7 RNA per mouse intramuscularly and challenged with $1x10^4$ TC-1 or P3 (A15) tumor cells per mouse via tail vein injection 2 weeks after vaccination. Depletions were started 1 week prior to tumor challenge. Monoclonal antibody (MAb) GK1.5 was used for CD4 depletion, MAb 2.43 was used for CD8 depletion, and MAb PK136 was used for NK1.1 depletion. Flow cytometry analysis revealed that 95% of the appropriate lymphocyte subset was depleted while other subsets were at normal levels. Depletion was terminated on day 21 after tumor challenge. The lung weight and number of pulmonary nodules in each mouse were evaluated and counted as described earlier.

五、結果與討論

Self-replicating SINrep5 RNA Vaccines Generate Significant Antitumor Effects against TC-1 P3 (A15) Cells

We have previously linked herpes simplex virus type 1 (HSV-1) protein VP22, to E7 in a naked RNA vaccine and found that it enhanced the E7-specific CD8+ T cell immune response and generated a potent antitumor effect against TC-1[12]. We therefore vaccinated C57BL/6 mice with various self-replicating SINrep5 RNA vaccines and challenged them with TC-1 or TC-1 P3 (A15) tumor cells as described in the **Materials and Methods** to determine whether these vaccines could control tumors with MHC class I downregulation. We found that there were fewer pulmonary nodules of TC-1 tumor cells in mice vaccinated with the self-replicating SINrep5-VP22/E7 RNA vaccine than in mice vaccinated with other RNA vaccines ($p<0.001$, one-way ANOVA) **(Figure 1A)**. We then investigated the antitumor effect generated by each of these vaccines following TC-1 P3 (A15) tumor challenge. Mice vaccinated with self-replicating SINrep5 RNA vaccines generated fewer pulmonary tumor nodules

($p<0.001$, one-way ANOVA) than the naïve mice **(Figure 1B)**. However, there was no significant difference in the mean pulmonary weight or the pulmonary tumor nodules between mice vaccinated with different self-replicating SINrep5 RNA vaccines, such as no insert, E7, VP22, or VP22/E7 **(Figure 1B)**. Our data reveal that self-replicating SINrep5 RNA vaccines can generate anti-tumor effects against tumors with MHC class I downregulation.

Different Subsets of Lymphocytes are Important for the Antitumor Effect against TC-1 or TC-1 P3 (A15) Tumor Cells Mediated by Self-Replicating SINrep5-VP22/E7 RNA Vaccine

To determine the subset of lymphocytes that are important for the antitumor effect generated by self-replicating SINrep5 RNA vaccines against TC-1 or TC-1 P3 (A15) tumor cells, we performed *in vivo* antibody depletion experiments. CD4, CD8, and NK1.1 depletions were initiated one week prior to TC-1 or TC-1 P3 (A15) tumor challenge in self-replicating SINrep5-vaccinated mice. As shown in **Figure 2A**, depletion of CD8+ T cells and NK1.1 cells resulted in similar mean numbers of pulmonary nodules after TC-1 challenge, significantly greater than those generated in the nondepleted or CD4 depleted group ($p<0.001$, one-way ANOVA). In comparison, in an experiment using mice challenged with TC-1 P3 (A15), NK-depleted mice had developed higher number of pulmonary tumor nodule ($p<0.001$, one-way ANOVA) than the other groups, such as non-depleted, CD4-depleted, or CD8-depleted mice **(Figure 2B)**. This was also observed in the mice vaccinated with self-replicating SINrep5 without insert challenged with TC-1 P3 (A15). These results indicated that while CD8+ T cells and NK cells were essential for the antitumor effect generated by the self-replicating SINrep5-VP22/E7 RNA vaccine against TC-1. However, only NK cells were important for the antitumor effect mediated by self-replicating SINrep5-VP22/E7 or SINrep5 RNA against TC-1 P3 (A15).

The TC-1 P3 preclinical tumor model serves as a suitable model for investigating mechanisms of immune evasion and developing immunotherapeutic strategies to control cancers that evade immunotherapy. The ability of neoplastic cells to evade the immune system remains a formidable barrier limiting the success of immunotherapy. Tumor cells can undergo a variety of other mechanisms to evade host defenses. Tumors may alter other surface molecules such as MHC class I and II [26] or co-stimulatory molecules to elude immune effector cells [27]. Defects in the antigen processing machinery of tumor cells play a role in the escape of tumors from recognition by T cells [28]. Deficiency in TAP allows tumors to evade immune surveillance [29]. Another evasion method is expression of Fas ligand on tumor cells, which can induce apoptosis of T cells [30]. Other mechanisms of immune evasion include altered expression of adhesion molecules [31] and dysregulation of cytokine

expression (i.e. secretion of immunosuppressive cytokines) such as transforming growth factor-β[32]. Downregulation of MHC class I represents one important mechanism of immune evasion. While 80% of the subclones derived from TC-1 P3 demonstrate downregulation of MHC class I molecules, 20% of these subclones show levels of MHC class I expression similar to TC-1[23]. Thus, it will be important to further characterize these subclones in order to obtain a more comprehensive understanding of immune evasion and to design vaccine strategies to overcome such evasion.

Our results indicate that self-replicating SINrep5 RNA vaccine can generate an impressive anti-tumor effect against murine tumors with downregulated MHC class I expression through the NK cells.

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七、圖解

Figure 1. Tumor protection mediated by various SINrep5 self-replicating RNA vaccines. Mice (five per group) were immunized intramuscularly with 1 μg of SINrep5-VP22, SINrep5-E7, SINrep5-VP22/E7, and SINrep5 (no insert) RNA per mouse. Two weeks after vaccination, mice were challenged with TC-1 or TC-1 P3 (A15) tumor cells via intravenous tail vein injection at a dose of $1x10^4$ cells/mouse. Mice were monitored twice a week and sacrificed at day 28 after tumor challenge. Lungs were dissected from mice 42 days after vaccination with the various RNA vaccines. The mean lung weight and the number of pulmonary tumor nodules were used as a measurement of the effectiveness of the various self-replicating RNA vaccines at controlling MHC I normal expressed (TC-1) or down-regulated TC-1 P3 (A15) HPV-16 E7-expressing tumor growth. **(A) Number of pulmonary tumor nodule in mice immunized with self-replicating SINrep5 RNA vaccines and challenged with TC-1.** There were fewer mean pulmonary nodules in mice vaccinated with the self-replicating VP22/E7 RNA vaccine than in mice vaccinated with the other RNA vaccines or naïve mice ($p<0.001$, one-way one-way ANOVA). SEM, standard error of the mean. **(B) Number of pulmonary tumor nodule in mice immunized with self-replicating SINrep5 RNA vaccines and challenged with P3-A15.** *Note*: There were fewer mean pulmonary nodules in mice vaccinated with the self-replicating RNA vaccines than naïve mice challenged with P3-A15. There was no significant differences of pulmonary tumor nodule in mice vaccinated with the self-replicating RNA vaccines.

Figure 2. Effect of lymphocyte subset depletions on the potency of self-replicating SINrep5 RNA vaccines. Mice were immunized with 1 μg of self-replicating SINrep5 with no insert or VP22/E7 RNA per mouse via intramuscular injection. Two weeks after vaccination, mice were challenged with 10^4 TC-1 or TC-1 P3 (A15) cells/mouse intravenously via tail vein. Depletions were initiated 1 week prior to tumor challenge

and terminated 21 days after tumor challenge. Four weeks after tumor challenge, mice were sacrificed. **(A) Number of pulmonary tumor nodule in mice immunized with self-replicating SINrep5 RNA vaccines and challenged with TC-1.** *Note*: Depletion of $CD8^+$ T cells and NK1.1 cells resulted in similar mean numbers of pulmonary tumor nodules, significantly greater than those generated in the nondepleted and CD4 depleted groups. SEM, standard error of the mean. **(B) Number of pulmonary tumor nodule in mice immunized with self-replicating SINrep5 RNA vaccines and challenged with P3-A15.** *Note:* There were greater mean pulmonary tumor nodule in NK-depleted mice vaccinated with the self-replicating SINrep5-VP22/E7 RNA vaccine challenged with TC-1 P3 (A15) than the other groups.

八、圖示

Figure 1

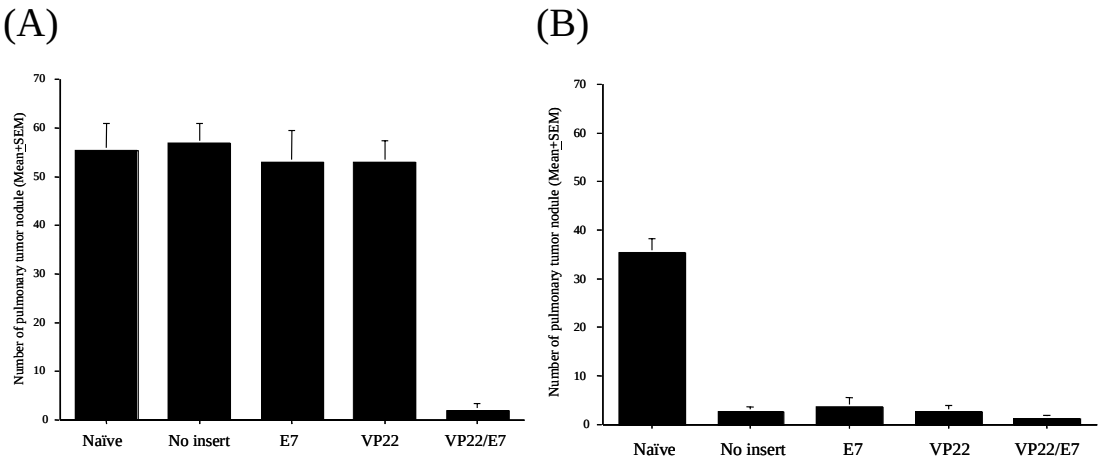

Figure 2