REPORT

A vaccine candidate against the human papillomavirus: an alternative to treat cervico-uterine tumors

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ABSTRACT

In this study, we obtained a vaccine against tumors generated by the human papillomavirus type 16 (HPV16), a current and highly relevant issue in the field of cancer immunotherapy. In contrast to some vaccines currently being tested in clinical trials against the HPV, of which none of them have been approved, the novelty of this result is the design of an original vaccine, based on a synthetic peptide spanning the minimal sequence of a cytotoxic T lymphocyte (CTL) epitope from the HPV tumor antigen E7, which was adjuvanted with VSSP (Very Small Size Proteoliposomes, Center for Molecular Immunology, Cuba). Its proof-of-concept in the murine HPV16 tumor model demonstrated the induction of $CD8^+$ T cells specific against the E7 epitope in mice treated with this vaccine candidate, as well as tumor regression and a significant increase in survival. This is the first report describing the induction of tumor regression by therapeutic immunization with a minimal CTL epitope and VSSP, and the first example of cancer immunotherapy by combining VSSP with a viral antigen. These results have been patented, presented in several international scientific conferences and published in "Vaccine" (Torréns I, Mendoza O, Batte A, Reyes O, Fernández LE, Mesa C, *et al*. Immunotherapy with CTL peptide and VSSP eradicated established human papillomavirus (HPV) type 16 E7-expressing tumors. Vaccine 2005;23:5768-74).

*I*ntroduction

Cervical cancer is the second cause of death by can-cer in women worldwide; the human papillomavirus (HPV), specifically type 16 (HPV16) being associated to it. More than 99% of cervical cancers and their precursor lesions are diagnosed with HPV DNA. The HPV oncogenic proteins E6 and E7 are relevant for the induction and persistence of cellular transformation, being co-expressed in most of the HPV-containing cervical cancers. Therefore, these oncogenic proteins represent target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated tumors [1].

In recent years, clinical trials have been carried out with several vaccines containing peptides restricted to the major histocompatibility complex type I (MHC-I) in patients with progressive malignancies associated to HPV and other types of tumors. However, the re-sults have been discouraging because of the induction of a weak T cell response, and therefore, an irrelevant clinical benefit.

Some studies describe different strategies to solve the failure of these peptide vaccines to induce a potent and sustained immunity, although they are still ineffective.

The novelty of this result is the design of an original vaccine, based on a synthetic peptide spanning the minimal sequence of a cytotoxic T lymphocyte (CTL) epitope from the HPV tumor antigen E7 that combined with VSSP (Very Small Size Proteoliposomes, Center for Molecular Immunology, Cuba) [2] was able to induce a potent T-cell response against pre-established tumors in the absence of oil-based components.

The aim of this study was to demonstrate our hypothesis that this type of peptide-based vaccine increases its immunogenicity and anti-cancer properties when combined with a complete adjuvant. Thus, our findings are highly original, with a novel approach for the development of therapeutic vaccines with the perspective of treating cancer [3]. These results were also patented [4].

This work consisted of several steps: chemical synthesis of the CTL peptide, establishing the TC 1 murine model and the demonstration of the HPV16 E7 protein expression in TC-1 cells and in animal tumors, evaluating protection against tumor challenge in a prophylactic setting of immunized animals, evaluating protection after a second tumor challenge, evaluating the complete regression of pre-established tumors in a therapeutic setting, evaluating survival in the vaccinated animals and assessing the immunogenicity of the vaccine candidate through IFN-γ ELISPOT and IL-10 ELISA assays.

*R*esults

Chemical synthesis of the CTL peptide

The peptide selected for the proof of concept was a H2-D^b-restricted CTL peptide in mice, corresponding to aas. 49-57 in the E7 oncoprotein of HPV16 (RAHYNIVTF), which was called E7(p).

The chemical synthesis of E7(p) was carried out on a solid phase; the E7(p) being further purified by high pressure liquid chromatography procedures at more than 95%. Peptide identity was confirmed by mass spectrometry, using the electro-spray ionization technique.

The peptide was stored at 4 ºC until use. It was dissolved in water for injection at 1 mg/mL just before

1. Granadillo M, Torréns I. Human papillomavirus vaccines: current status and perspectives. Biotecnol Apl. 2008; 25:XX.

2. Mesa C, De León J, Rigley K, Fernández LE. Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for Th1 induction and dendritic cell activation. Vaccine 2004; 22:3045-52.

3. Torréns I, Mendoza O, Batte A, Reyes O, Fernández LE, Mesa C, *et al*. Immunotherapy with CTL peptide and VSSP eradicated established human papillomavirus (HPV) type 16 E7-expressing tumors. Vaccine 2005; 23:5768-74.

4. Torréns I, Guillén G, Pajón R, Reyes O, Fernández LE inventores; CIGB solicitante. Composiciones farmacéuticas que contienen antigenos del virus de papiloma humano. AR045815 (A1). 2005 Nov 16.

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application. Fifty micrograms of the E7(p) peptide was then mixed with 160 µg of VSSP to a volume of 0.2 mL; the E7(p)+VSSP vaccine dose was ready to be administered to the experimental animals.

Establish of TC-1 murine tumoral model. Demonstration of expression of HPV E7 protein in TC-1 cells and animal tumors

We used the TC-1 murine tumor model, consisting of immunocompetent C57BL/6 mice which were challenged with TC-1 tumor cells. The TC-1 tumor cell line, expressing the HPV16 E7 protein, derives from a primary culture of C57BL/6 mice lung cells immortalized and transformed with HPV16 E6 and E7 genes and a human activated c-Hras gene [5]. This tumor cell line was kindly provided by Dr. TC Wu (The Johns Hopkin Medical Institute, Baltimore, Md., USA). It has been widely used in the proof-of-concept of several vaccine preparations developed in laboratories throughout the world.

The actions for its establishment in our laboratory were: (i) to make the tumor cell line syngeneic with C57BL/6 mice purchased from the National Center for the Production of Laboratory Animals (CENPALAB, Cuba); (ii) the demonstration of HPV16 E7 protein expression by reverse transcription and polymerase chain reaction and Western blot procedures in cells and tumors isolated from these mice; and (iii) repeated experiments of tumor inoculation, followed by the analysis of tumor implantation to obtain homogeneous and repetitive results.

Prophylactic evaluation of protection against tumor challenge of animals immunized with the vaccine candidate

In this first assay, C57BL/6 mice were subcutaneously immunized in the right flank with two doses given 14 days apart. Seven days after the second immunization, the animals were challenged with $5x10^4$ TC-1 tumor cells administered subcutaneously in the right hind limb. Three additional control groups were included in the study, receiving: VSSP, E7(p) + incomplete Freund´s adjuvant (IFA) and phosphate buffered saline (PBS), respectively. The results indicated that mice immunized with the vaccine candidate were protected against the tumor challenge ($P < 0.0001$) (Table 1).

Evaluation of protection after a second tumor challenge

Five surviving animals, immunized with E7(p) + VSSP from the previous experiment, were chosen to evaluate the longevity of the immune response against the HPV. They were subcutaneously re-challenged with 2×10^5 TC-1 cells in the left hind limb, fifty days after the first tumor challenge. A group of five naive mice from the same lot of animals were included as the control, being challenged as well. Results indicated a 100% protection against tumor implantation in vaccinated mice, while all the control animals showed measurable tumors 21 days after challenge.

Table 1. Results of tumor implantation in immunized mice

Group	Incidence of tumor / Total	Treatment
I	10/10	50 µg E7(p) + IFA
II	2/10	50 µg E7(p) + VSSP
III	10/10	VSSP
IV	10/10	Placebo (PBS)

Therapeutic evaluation of complete regression in established tumors

In the therapeutic setting, mice were previously inoculated with 2×10^5 TC-1 cells. Ten days after inoculation all animals showed measurable subcutaneous tumors, with volumes in the range of 400-500 mm^3. Then, mice were randomly distributed in three different groups (ten mice each) and administered with 2 doses of the vaccine preparation at a 14 day interval. After 50 days, when mice treated with E7(p) + IFA, VSSP or PBS showed a 100% tumor incidence, the therapeutic immunization with E7(p) + VSSP induced tumor regression in all the animals treated (Figure 1a).

Evaluation of survival in animals treated with the vaccine candidate

Mice were observed for 100 days after treatment to determine the effects of the E7(p) + VSSP therapy on survival. The Kaplan-Meier plot (Figure 1b) shows survival results of 80% for the group treated with E7(p) + VSSP (8/10), a result that is statisti-

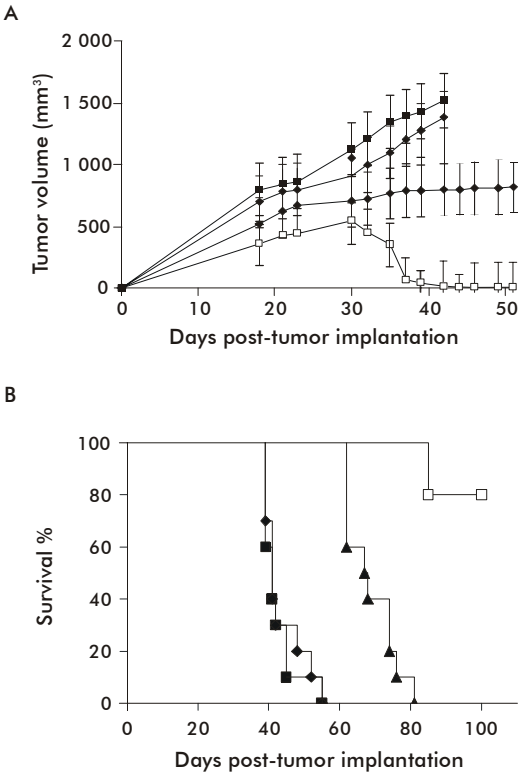

Figure 1. The therapeutic immunization with the E7(p) + VSSP induces TC-1 tumor regression and long-term survival. (a) C57BL/6 mice (10 per group) were inoculated with 2×10^5 TC-1 cells in a hind limb. Ten and 24 days after tumor graft, mice were immunized with PBS (■), VSSP (◆), E7(p)+IFA (▲), or E7(p)+ VSSP (□). Tumor volume was measured for 50 days. (b) Mice were observed and survival recorded for 100 days.

5. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996; 56:21-6.

cally highly significant when compared to the groups treated with E7(p) + IFA, VSSP or PBS, respectively ($P < 0.0001$).

Immunogenicity of the vaccine candidate as assessed by IFN-γ ELISPOT and ELISA assays

As an initial step to identify the effector mechanisms involved in tumor eradication, the immune response of cytokine secretion induced by immunizing with E7(p) + VSSP was characterized by an ELISA assay. An anti-IFN-γ ELISPOT assay was carried out to compare the immune response mediated by anti-E7 specific CTLs developed in the different groups of vaccinated mice. Splenocytes from mice immunized with the E7(p) + VSSP secreted high levels of IFN-γ in response to the stimulation with the peptide, and low levels of IL-10. These were indicators of a T helper lymphocyte type 1 response. The highest number of IFN-γ-secreting cells was induced in mice immunized with E7(p) + VSSP, suggesting the induction of a cytotoxic $CD8^+$ T cell response (Figure 2).

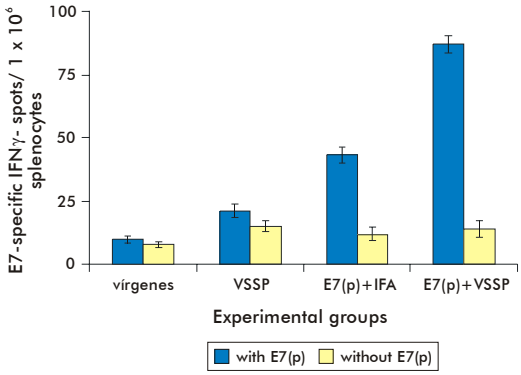

Figure 2. Demonstration of the induction of specific $CD8^+$ T lymphocyte precursors against the E7 protein in C57BL/6 mice immunized with various immunogens, as assessed by IFN-γ ELISPOT assay. Mice (three per group) were immunized twice with VSSP, E7(p) + IFA or E7(p) + VSSP, and other remain naïve. Splenocytes from each mouse were isolated 7 days after the last immunization. The number of precursor T cells specific for the E7 protein and producing IFN-γ was determined in the presence (solid bars) or absence (open bars) of E7(p).