A novel strategy to improve antigen presentation for active immunotherapy in cancer. Fusion of the human papillomavirus type 16 E7 antigen to a cell penetrating peptide

Milaid Granadillo1, Isis Torrens1, Maribel Guerra1, Aileen Batte1, Yordanka Soria2, Osmani Mendoza2, Aracelys Blanco3, Alexis Musacchio3, Victoria M Lugo4

1 Departamento de Cáncer, División de Farmacéuticos, Dirección de Investigaciones Biomédicas, 2 Biotério, 3 Departamento de Proteómica, División de Química Física, Dirección de Investigaciones Biomédicas y 4 Dirección de Desarrollo Tecnológico, Centro de Ingeniería Genética y Biotecnología, CIGB Ave. 31 e/ 158 y 190, Cubanacán, Playa, AP 6162, CP 10600, La Habana, Cuba
E-mail: milaid.granadillo@cigb.edu.cu

ABSTRACT

Facilitating the delivery of exogenous antigens to antigen-presenting cells, ensuing processing and presentation via the major histocompatibility complex class I and induction of an effective immune response are fundamental for an effective therapeutic cancer vaccine. In this regard, we propose the use of cell-penetrating peptides fused to a tumor antigen. To demonstrate this concept we designed a fusion protein comprising a novel cell-penetrating and immunostimulatory peptide corresponding to residues 32 to 51 of the Limulus anti-lipopolysaccharide factor protein (LALF32-51), linked to human papillomavirus 16 E7 antigen (LALF32-51-E7). In this work, we demonstrated that the immunization with LALF32-51-E7 using the TC-1 mouse model induces a potent and long-lasting anti-tumor response supported on an effective E7-specific CD8+ T-cell response. The finding that therapeutic immunization with LALF32-51 or E7 alone, or an admixture of LALF32-51 and E7, does not induce significant tumor reduction indicates that covalent linkage between LALF32-51 and E7 is required for the anti-tumor effect. These results support the use of this novel cell-penetrating peptide as an efficient means for delivering therapeutic targets into cellular compartments with the induction of a cytotoxic CD8+ T lymphocyte immune response. This approach is promissory for the treatment of tumors associated with the human papillomavirus 16, which is responsible for the 50% of cervical cancer cases worldwide and other malignancies. Furthermore, protein-based vaccines can circumvent the major histocompatibility complex specificitiy limitation associated with peptide vaccines providing a greater extent in their application.

Keywords: fusion protein, E7, cell-penetrating peptides, LALF32-51, human papillomavirus

RESUMEN

Nueva estrategia para mejorar la presentación antigénica en la inmunoterapia activa del cáncer. Fusión del antígeno E7 del virus del papiloma humano tipo 16 a un péptido penetrador a células. Facilitar la internalización de moléculas exógenas por las células presentadoras de antígenos, su procesamiento, presentación en el complejo mayor de histocompatibilidad tipo I y la inducción de una respuesta inmune efectiva, constituyen premisas fundamentales en el diseño de candidatos vacunales terapéuticos contra cáncer. Para ello se propone el uso de péptidos penetradores a células (PPC) fusionados con el antígeno tumoral. Para demostrarlo se empleó un novedoso PPC derivado de la proteína factor antilipopolisacárido de Limulus (LALF32-51), fusionado genéticamente a una muteína de la proteína E7 del virus del papiloma humano tipo 16 (VPH16), que denominamos LALF32-51-E7. En este estudio se demuestra que la inmunización con LALF32-51-E7, en el modelo murino tumoral TC-1 de VPH16, induce una respuesta antitumoral potente, protectora y de larga duración, cuyo mecanismo es la inducción de una respuesta celular mediada por linfocitos T citotóxicos CD8+ específicos contra el antígeno E7. La inmunización con la E7 sola o la mezcla de ella con el PPC no reproduce los efectos obtenidos con la fusión covalente LALF32-51-E7. Los resultados avalan este nuevo PPC como una herramienta atractiva para la internalización de antígenos con la consecuente inducción de una respuesta de linfocitos T citotóxicos CD8+. Además, abre una perspectiva promisoria para el tratamiento de tumores asociados al VPH16 responsable del 50% del cáncer cervical y de otras tumores. A diferencia de las vacunas peptídicas, las basadas en proteínas no están restringidas por el antígeno leucocitario humano y permiten una amplia aplicación.

Palabras clave: proteína de fusión, E7, péptidos de penetración celular, LALF32-51, virus del papiloma humano

Introduction

The persistent infection with human papillomavirus (HPV), particularly type 16 (HPV16), is associated with the development of malignant lesions of the oral and genital tract [1]. Cervical cancer is the second cause of women cancer mortality worldwide and the infection with HPV16 accounts for about 50% of all cases each year. Currently, there are two licensed prophylactic HPV vaccines, but their benefits might be visible only after decades. The prohibitive costs and the limited number of HPV types included in these vaccines, and the fact that do not generate therapeutic effects against established HPV infections have encouraged researchers to develop therapeutic vaccines for the control of existing HPV infection and associated malignancies.

The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular
transformation and are co-expressed in most HPV-containing cervical cancers [2]. Therefore, these oncogenic proteins represent ideal target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated tumors [3]. Unfortunately, exogenous proteins are weak immunogens, typically inducing humoral immune responses while a strong tumor-specific cytotoxic T-lymphocyte (CTL) response is necessary for a successful cancer vaccine. Therefore, strategies employing adjuvants and fusion with immunostimulatory molecules or more recently the use of cell-penetrating peptides (CPP) are overcoming this problem. Today, various therapeutic vaccines against HPV infections are in clinical trials, but none yet approved for marketing.

The novelty of this result is the design of an original vaccine (LALF32-51-E7 fusion protein) with promising perspectives to treat HPV16 related malignancies, based on the covalent linkage of a novel CPP with immunostimulatory properties, the peptide from Limulus polyphemus anti-lipopolysaccharide factor protein LALF32-51 and the HPV16 E7 mutein.

In this work, we asked whether LALF32-51 would be capable of delivering biologically-active proteins to the cytoplasmic compartment via the plasma membrane and if targeting a viral oncoprotein to the cytoplasmic compartment could enhance a tumor-specific immune response. It was demonstrated that LALF32-51 penetrates the cell membrane and delivers E7 into cells. In a preclinical model of HPV16-induced cervical carcinoma, vaccination with adjuvant-free LALF32-51-E7 fusion protein significantly improves the presentation of E7-derived peptides to T cells in vitro and induces suppression of tumor growth [4]. The current findings are original and constitute a promising approach in the development of cancer therapeutic vaccines. This research was granted the 2011 Award of the Cuban National Academy of Sciences.

**Results**

**Cloning, expression and purification of LALF32-51-E7 fusion protein**

The HPV16 E7 gene and LALF32-51 were chemically synthesized as double-stranded DNA. The HPV16 E7 sequence contained a base substitution of T by G in the triplet encoding the first cysteine, in order to disrupt the binding site of E7 to protein Rb. Both DNA synthetic fragments were ligated to pM238 Escherichia coli expression vector [5]. The resulting plasmid encodes a C-terminal histidine-tagged fusion protein consisting of LALF32-51 linked at its C-terminus to the HPV16 E7 mutein, abbreviated LALF32-51-E7. To improve safety, and since the ampicillin resistance gene is precluded for use in humans, the kanamycin resistance gene was introduced as a selectable marker of the final expression vector pPEPE7M-7K.

The E. coli strain BL21 (DE3) was used as host for recombinant protein production. BL21 (DE3) cells harboring pPEPE7M-7K were grown in a 5-L bioreactor, the cellular biomass being further disrupted in a French press. After centrifugation, the pellet was recovered and solubilized in 6 M urea. The soluble fraction was purified using a His-Select™ Nickel Affinity Gel and standard immobilized metal ion affinity chromatography procedures. The eluted fraction was further loaded onto a HiPrep 26/10 desalting column. Finally, the fusion protein was filter-sterilized (0.2 µm pore size) and stored at -20 °C until use. LALF32-51-E7 was highly expressed in *E. coli* (18%) and easily purified with a single affinity chromatographic step with a high purity (90%), yielding 197 mg/L of bacterial culture.

**Characterization of LALF32-51-E7 fusion protein assessed by different methodologies**

LALF32-51-E7 fusion protein was characterized by size exclusion analytic HPLC, sodium dodecyl sulfate polyacrylamide electrophoresis under reducing and non-reducing conditions, Western Blot, transmission electron microscopy and mass spectrometry. The fusion protein was obtained in a highly aggregated form, a property that is considered very important to increase the immunogenicity of an antigen preparation. The identity of LALF32-51-E7 was verified by mass spectrometry.

**Evaluation of anti-tumor response generated by therapeutic immunization with LALF32-51-E7 in TC-1 murine model**

In these experiments, we used the murine H-2b tumor cell line TC-1 (containing the HPV16 E6, E7 and E8 gene products). Vaccination with adjuvant-free LALF32-51-E7 fusion protein significantly improves the immune response. It was demonstrated that LALF32-51-E7 fusion protein has the ability to penetrate into the cells. The cell-penetrating ability of LALF32-51 was demonstrated by transmission electron microscopy using peripheral blood mononuclear cells (Figure 1), immunofluorescence microscopy in several cell lines (J774, CaSkii, HeLa, TC-1, SiHa) and confocal microscopy using murine splenocytes. The ability of LALF32-51-E7 fusion protein to penetrate into cells was demonstrated by immunofluorescence microscopy and Western Blot using J774 murine macrophages cell line and by confocal microscopy using murine splenocytes (Figure 2). Taking into account these results, E7 protein is only detected inside the cells when E7 is fused to LALF32-51 peptide.

![Figure 1](image-url)

**Figure 1.** The fusion LALF32-51-E7 is able to penetrate into the cells. A) Cell-penetrating ability of LALF32-51-E7 fusion protein was assessed by different methodologies. LALF32-51-E7 fusion protein was characterized by size exclusion analytic HPLC, sodium dodecyl sulfate polyacrylamide electrophoresis under reducing and non-reducing conditions, Western Blot, transmission electron microscopy and mass spectrometry. The fusion protein was obtained in a highly aggregated form, a property that is considered very important to increase the immunogenicity of an antigen preparation. The identity of LALF32-51-E7 was verified by mass spectrometry.
activated human Ha-ras genes) that was kindly provided by Dr. TC Wu (Johns Hopkins University, Baltimore) and maintained as previously described [6].

To determine if a LALF\textsubscript{32-51}-E7 vaccine could induce regression of established TC-1 tumors and if the covalent linkage between LALF\textsubscript{32-51} and E7 could be relevant to anti-tumor response, female C57Bl/6 mice (ten per group) were injected subcutaneously with \(2 \times 10^5\) TC-1 cells in the leg (day 0). Subsequently, on days 12 and 19 they were treated with phosphate-buffered saline (PBS), \(30 \mu g\) of LALF\textsubscript{32-51}-E7, molar equivalents of LALF\textsubscript{32-51} alone, E7 alone or a mixture of LALF\textsubscript{32-51} and E7 (LALF\textsubscript{32-51} + E7). By the end of the 30-day observation period (Figure 3A), only LALF\textsubscript{32-51}-E7 vaccination induced a suppression of tumor growth. Therapeutic immunization with LALF\textsubscript{32-51} alone, or E7 alone, or a mixture of LALF\textsubscript{32-51} and E7, does not induce significant tumor reduction indicates that covalent linkage between LALF\textsubscript{32-51} and E7 is required for the anti-tumor effect.

Given the ability of therapeutic LALF32-51-E7 immunization to induce a significant reduction of TC-1 tumors, the correlation between antigen dose and anti-tumor responses was examined. In these studies, therapy was initiated 14 days post-tumor implantation, when 100% of mice had palpable subcutaneous tumors. Female C57Bl/6 mice (ten per group) were injected subcutaneously with \(2 \times 10^5\) TC-1 cells in the leg (day 0), then they were treated with PBS or either 30, 60 or \(120 \mu g\) of LALF\textsubscript{32-51}-E7 on days 14 and 21. By the end of the 30-day observation period (Figure 3B), the immunization with 60 and \(120 \mu g\) of LALF\textsubscript{32-51}-E7 resulted statistically significant in the reduction of tumor volumes compared to 30 \(\mu g\) LALF\textsubscript{32-51}-E7 immunization. Considering that no statistical differences observed of tumor-volume reduction between the doses of 60 and 120 \(\mu g\) of LALF\textsubscript{32-51}-E7, we chose 60 \(\mu g\) of LALF\textsubscript{32-51}-E7 as the optimal dose to generate potent anti-tumor responses against E7 expressing tumors in mice.

Based on the fact that two immunizations in a therapeutic setting with the protein LALF\textsubscript{32-51}-E7 induce a significant tumor volume reduction, we investigated the effect of administering either three or four doses of this antigen. Female C57Bl/6 mice (ten per group) were injected subcutaneously with \(5 \times 10^4\) TC-1 cells in the leg (day 0). Thirteen days post-tumor implantation, when 100% of mice had palpable subcutaneous

Figure 2. Confocal microscopy analysis in murine splenocytes of the cell-penetrating ability of LALF32-51-E7 fusion protein. The splenocytes were incubated with 1.66 \(\mu M\) of LALF\textsubscript{32-51} biotinylated peptide, LALF\textsubscript{32-51}-E7 or E7, respectively, or PBS for 10 min. Finally, slides were observed with a 60 x objective on a Nikon microscope with attached laser confocal scanning system MRC 600. Green fluorescent staining indicates peptide or protein localization and red staining corresponds to propidium iodide-labeled nuclei.

Figure 3. Therapeutic immunization with LALF\textsubscript{32-51}-E7 induces a significant tumor volume reduction that is dose dependent. C57Bl/6 mice (10 per group) were subcutaneously injected with \(2 \times 10^5\) TC-1 cells in the right leg (0) and monitored until tumor was apparent in all animals. A) At 12 and 19 days post-implantation, mice were immunized with PBS, 30 \(\mu g\) LALF\textsubscript{32-51}-E7 and molar equivalents of LALF\textsubscript{32-51} alone, E7 alone or LALF\textsubscript{32-51} + E7. Tumor volumes of subcutaneous nodules were monitored for 30 days until the control mice began to die. Vaccination with LALF\textsubscript{32-51}-E7 fusion protein induced suppression of tumor growth that was statistically significant compared with the other treatments groups (Mann Whitney, \(p < 0.001\)). B) Mice were immunized at 14 and 21 days post-tumor implantation with PBS, 30 \(\mu g\), 60 \(\mu g\) and 120 \(\mu g\) LALF\textsubscript{32-51}-E7, respectively. Tumor volumes were monitored 30 days until the control mice became moribund. The immunization with 60 \(\mu g\) or 120 \(\mu g\) LALF\textsubscript{32-51}-E7 induced a significant reduction in tumor volumes with respect to the group receiving 30 \(\mu g\) LALF\textsubscript{32-51}-E7 (Mann Whitney, \(p < 0.01\)). In both graphics, data are presented as mean tumor volume (mm\(^3\)) ± standard deviation in the different groups of immunized mice and arrows indicate the treatment time point.
tumor, a regimen of two, three or four immunizations of 60 μg of LALF32-51-E7 or PBS was conducted at 7-day interval. By the end of the 43-day observation period, the therapeutic anti-tumor activity induced by two immunizations with LALF32-51-E7 was comparable to that induced by three or four immunizations with this protein.

**Evaluation of protection against tumor challenge in C57Bl/6 mice immunized with LALF32-51-E7**

To examine the ability of LALF32-51-E7 immunization to confer protection against in vivo challenge with TC-1 cells in a prophylactic setting, female C57Bl/6 mice (ten per group) were immunized subcutaneously either two, three or four times with 60 μg of LALF32-51-E7 or PBS at 14-day interval. Thirty days after the last immunization, mice were challenged with 2 × 10^4 TC-1 cells in the right leg followed by an initial period of 21 days. LALF32-51-E7 vaccination induced a potent suppression of tumor growth and the effect of two immunizations was comparable to that induced by three or four immunizations with this protein. On day 148 after the first challenge, the tumor-free animals from the LALF32-51-E7 vaccinated groups (five per group) were re-challenged with a larger dose of TC-1 cells (2 × 10^6 cells) in the left leg and observed for an additional 60-day period. In addition, a new group of untreated mice was challenged with tumor cells on day 148 to verify the tumorigenicity of the TC-1 cells. The 100% (5/5) of the untreated mice were moribund by day 55. In contrast, survival in the LALF32-51-E7 vaccinated group, that received two immunizations, was 60% (3/5) over this 60-day period, similar to the groups receiving three or four immunizations with the vaccine (p = 0.8985 and p = 0.7987, respectively).

According to the results obtained in both therapeutic and prophylactic setting, two doses of 60 μg of LALF32-51-E7 are sufficient to induce a potent and long-lasting anti-tumor response that can protect mice from tumor challenge.

**Evaluation of anti-tumor response generated by the therapeutic immunization with LALF32-51-E7 co-administered with different adjuvants**

Given the ability of therapeutic LALF32-51-E7 immunization to induce TC-1 tumor volume reduction, we evaluated if the co-administration of LALF32-51-E7 with different adjuvants could induce the regression of established TC-1 tumors and promote long-term survival of the animals. In these studies, therapy was initiated 14 days after tumor implantation, when 100% of mice had palpable subcutaneous tumors. Female C57Bl/6 mice (ten per group) were injected subcutaneously with 5 × 10^5 TC-1 cells in the leg (day 0). Subsequently, on days 14 and 21 they were treated with either PBS, 60 μg of LALF32-51-E7 alone or mixed with 120 μg of the Neisseria meningitidis very small size proteoliposome adjuvant (LALF32-51-E7 + VSSP), 30 μg of N. meningitidis outer membrane vesicles (LALF32-51-E7 + OMV) or 90 μg of QuilA (LALF32-51-E7 + QuilA). By the end of the 32-day observation period, the therapeutic anti-tumor activity induced by LALF32-51-E7 alone was comparable to that induced by LALF32-51-E7 co-administered with VSSP, OMV or QuilA as adjuvants.

To determine the effect of LALF32-51-E7 mixed with different adjuvants on long-term survival, the animals were observed for a period of 110 days. Compared with the LALF32-51-E7-treated mice, only the group immunized with LALF32-51-E7 + VSSP showed a statistically significant survival rate over this 110-day observation period (p = 0.0386).

**Immunogenicity of the vaccine candidate as assessed by LDH-cytotoxicity assay and IFN-γ ELISPOT**

As an initial step to identify the effectors mechanisms involved in the anti-tumor response generated by the immunization with LALF32-51-E7, we evaluated the cellular immune response by LDH-cytotoxicity assay and IFN-γ ELISPOT. The results obtained by LDH-cytotoxicity assay shown that the CTL activity induced by LALF32-51-E7 immunization is dose dependent. The effectors cells derived from mice immunized with 60 μg of LALF32-51-E7 were significantly more cytotoxic than those from mice immunized with 30 μg of LALF32-51-E7. The highest ELISPOT numbers were found in mice receiving LALF32-51-E7 alone. The number of IFN-γ-secreting splenocytes in mice immunized with LALF32-51-E7 alone was statistically significant and approximately up to eight-fold higher than the rest of the immunized groups (p < 0.001).

**Relevance of the study**

The relevance of this result is the design of an original vaccine (LALF32-51-E7 fusion protein) with promissory perspectives to treat HPV16 related malignancies, based on the covalent linkage of a novel CPP with immunostimulatory properties (LALF32-51 peptide from L. polyphemus) and HPV16 E7 mutein. Our data underline the efficacy of this approach at inducing broad immune responses in vivo, and offer a new strategy that could improve subunit cancer vaccine in a clinical setting.

**Conclusions**

We provide evidences that the fusion of protein E7 to LALF32-51 allows it to enter cells and thereby significantly improves the presentation of E7-derived peptides to T-cells in vitro. The LALF32-51-E7 fusion protein was also found to be a potent immunogen in vivo when injected in the absence of adjuvant, confirming the immunostimulatory capacity of LALF32-51. Furthermore, fusion to LALF32-51 enhances the therapeutic anti-tumor activities of viral protein-based vaccine.

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