REPORT

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

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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 ($LALF_{32-51}$) linked to human papillomavirus 16 E7 antigen ($LALF_{32-51}$-E7). In this work, we demonstrated that the immunization with $LALF_{32-51}$-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 $LALF_{32-51}$ or E7 alone, or an admixture of $LALF_{32-51}$ and E7, does not induce significant tumor reduction indicates that covalent linkage between $LALF_{32-51}$ 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 specificity limitation associated with peptide vaccines providing a greater extent in their application.

Keywords: fusion protein, E7, cell-penetrating peptides, $LALF_{32-51}$, human papillomavirus

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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* ($LALF_{32-51}$), fusionado genéticamente a una muteína de la proteína E7 del virus del papiloma humano tipo 16 (VPH16), que denominamos $LALF_{32-51}$-E7. En este estudio se demuestra que la inmunización con $LALF_{32-51}$-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 $LALF_{32-51}$-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 otros 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, $LALF_{32-51}$, virus del papiloma humano

*I*ntroduction

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

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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 ($LALF_{32-51}$-E7 fusion protein) with promissory 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 $LALF_{32-51}$, and the HPV16 E7 mutein.

In this work, we asked whether $LALF_{32-51}$ 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 $LALF_{32-51}$ penetrates the cell membrane and delivers E7 into cells. In a preclinical model of HPV16-induced cervical carcinoma, vaccination with adjuvant-free $LALF_{32-51}$-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 $LALF_{32-51}$-E7 fusion protein

The HPV16 E7 gene and $LALF_{32-51}$ 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 $LALF_{32-51}$ linked at its C-terminus to the HPV16 E7 mutein, abbreviated $LALF_{32-51}$-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 ºC until use. $LALF_{32-51}$-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 $LALF_{32-51}$-E7 fusion protein assessed by different methodologies

$LALF_{32-51}$-E7 fusion protein was characterized by size exclusion analytic HPLC, sodium dodecyl sulfate polyacrilamide electrophoresis under reducing and non-reducing conditions, W*estern 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 $LALF_{32-51}$-E7 was verified by mass spectrometry.

$LALF_{32-51}$-E7 fusion protein has the ability to penetrate into the cells

The cell-penetrating ability of $LALF_{32-51}$ was demonstrated by transmission electron microscopy using peripheral blood mononuclear cells (Figure 1), immunofluorescence microscopy in several cell lines (J774, CaSki, HeLa, TC-1, SiHa) and confocal microscopy using murine splenocytes. The ability of $LALF_{32-51}$-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 $LALF_{32-51}$ peptide.

Evaluation of anti-tumor response generated by therapeutic immunization with $LALF_{32-51}$-E7 in TC-1 murine model

In these experiments, we used the murine H-2^b tumor cell line TC-1 (containing the HPV16 E6, E7 and

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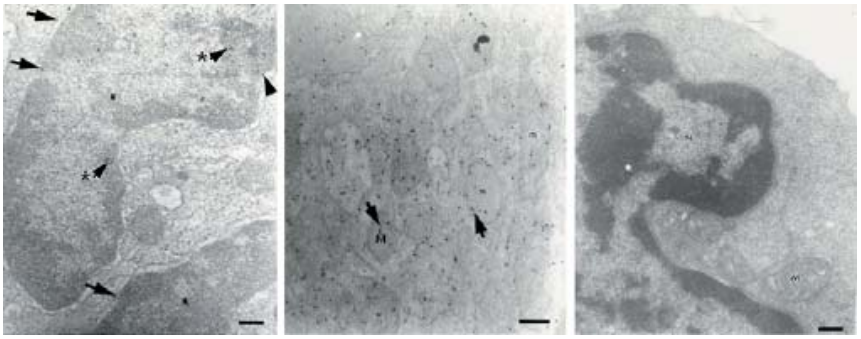

Figure 1. The fusion $LALF_{32-51}$-E7 is able to penetrate into the cells. A) Cell-penetrating ability of $LALF_{32-51}$ peptide was analyzed by transmission electron microscopy. Peripheral blood mononuclear cells were incubated with 339 μM of $LALF_{32-51}$ biotinylated peptide (A, B) or in absence of peptide (C) for 30 min. The samples were examined with JEOL/JEM 2000 EX transmission electron microscope. The colloidal gold particles are indicated by arrows. The peptide was detected in the perinuclear region (A, arrows), in the nuclear chromatin (A, arrows with asterisks) and inside the mitochondria (B, arrows). N: nucleus; M: mitochondrion. Bar equivalent to 200 nm.

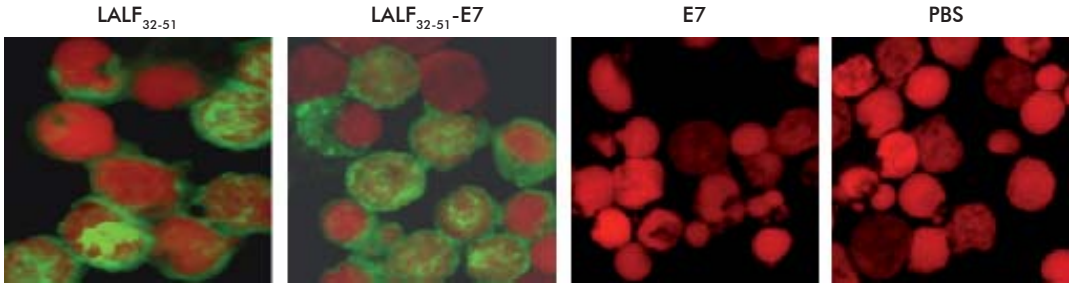

Figure 2. Confocal microscopy analysis in murine splenocytes of the cell-penetrating ability of $LALF_{32-51}$-E7 fusion protein. The splenocytes were incubated with 1.66 μM of $LALF_{32-51}$ biotinylated peptide, $LALF_{32-51}$-E7 or E7, respectively, or PBS for 10 min. Finally, slides were observed 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.

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 $LALF_{32-51}$-E7 vaccine could induce regression of established TC-1 tumors and if the covalent linkage between $LALF_{32-51}$ and E7 could be relevant to anti-tumor response, female C57Bl/6 mice (ten per group) were injected subcutaneously with 2×10^5 TC-1 cells in the leg (day 0). Subsequently, on days 12 and 19 they were treated with phosphate-buffered saline (PBS), 30 µg of $LALF_{32-51}$-E7, molar equivalents of $LALF_{32-51}$ alone, E7 alone or a mixture of $LALF_{32-51}$ and E7 ($LALF_{32-51}$ + E7). By the end of the 30 day observation period (Figure 3A), only $LALF_{32-51}$-E7 vaccination induced a suppression of tumor growth. Therapeutic immunization with $LALF_{32-51}$ or E7 alone, or a mixture of $LALF_{32-51}$ and E7, does not induce significant tumor reduction indicates that covalent linkage between $LALF_{32-51}$ 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 2×10^5 TC-1 cells in the leg (day 0), then they were treated with PBS or either 30, 60 or 120 µg of $LALF_{32-51}$-E7 on days 14 and 21. By the end of the 30-day observation period (Figure 3B), the immunization with 60 and 120 µg of $LALF_{32-51}$-E7 resulted statistically significant in the reduction of tumor volumes compared to 30 µg $LALF_{32-51}$-E7 immunization. Considering that no statistical differences observed of tumor-volume reduction between the doses of 60 and 120 µg of $LALF_{32-51}$-E7, we chose 60 µg of $LALF_{32-51}$-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 $LALF_{32-51}$-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 5×10^4 TC-1 cells in the leg (day 0). Thirteen days post-tumor implantation, when 100% of mice had palpable subcutaneous

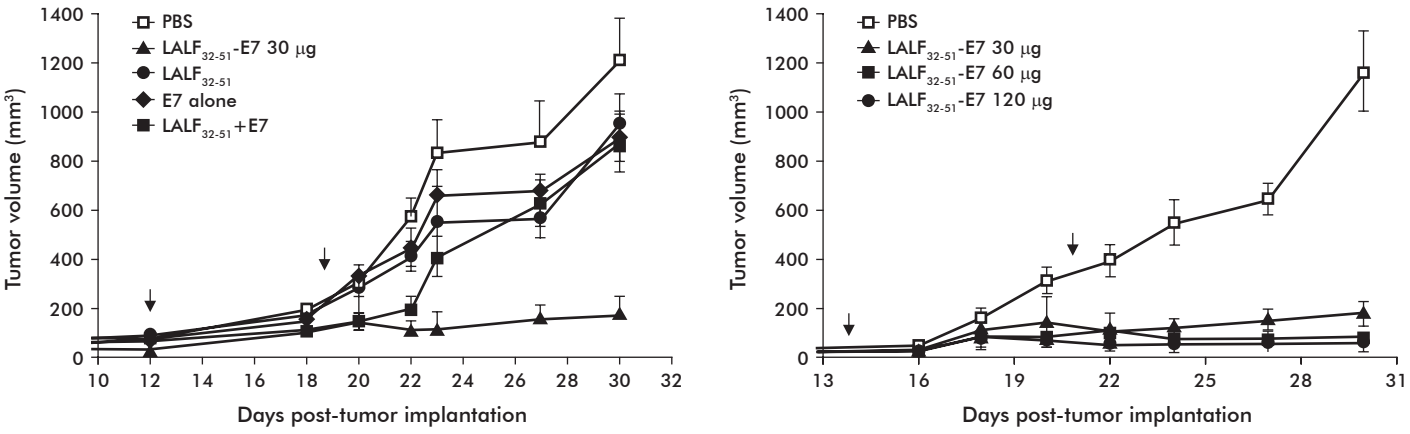

Figure 3. Therapeutic immunization with $LALF_{32-51}$-E7 induces a significant tumor volume reduction that is dose dependent. C57Bl/6 mice (10 per group) were subcutaneously injected with 2×10^5 TC-1 cells in the right leg (0) and monitored until tumor was apparent in all animals. A) At 12 and 19 days post-implantation, mice were immunized with PBS, 30 μg $LALF_{32-51}$-E7 and molar equivalents of $LALF_{32-51}$ alone, E7 alone or $LALF_{32-51}$ + E7. Tumor volumes of subcutaneous nodules were monitored for 30 days until the control mice began to die. Vaccination with $LALF_{32-51}$-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 μg, 60 μg and 120 μg $LALF_{32-51}$-E7, respectively. Tumor volumes were monitored 30 days until the control mice became moribund. The immunization with 60 μg or 120 μg $LALF_{32-51}$-E7 induced a significant reduction in tumor volumes with respect to the group receiving 30 μg $LALF_{32-51}$-E7 (Mann Whitney, $p < 0.01$). In both graphics, data are presented as mean tumor volume (mm3) ± 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 $LALF_{32-51}$-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 $LALF_{32-51}$-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 $LALF_{32-51}$-E7

To examine the ability of $LALF_{32-51}$-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 $LALF_{32-51}$-E7 or PBS at 14-day interval. Thirty days after the last immunization, mice were challenged with 2×10^5 TC-1 cells in the right leg followed by an initial period of 21 days. $LALF_{32-51}$-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 $LALF_{32-51}$-E7 vaccinated groups (five per group) were re-challenged with a larger dose of TC-1 cells (2×10^5 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 $LALF_{32-51}$-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 $LALF_{32-51}$-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 $LALF_{32-51}$-E7 co-administered with different adjuvants

Given de ability of therapeutic $LALF_{32-51}$-E7 immunization to induce TC-1 tumor volume reduction, we evaluated if the co-administration of $LALF_{32-51}$-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 5×10^4 TC-1 cells in the leg (day 0). Subsequently, on days 14 and 21 they were treated with either PBS, 60 μg of $LALF_{32-51}$-E7 alone or mixed with 120 μg of the *Neisseria meningitidis* very small size proteoliposome adjuvant ($LALF_{32-51}$-E7 + VSSP), 30 μg of *N. meningitidis* outer membrane vesicles ($LALF_{32-51}$- E7 + OMV) or 90 μg of QuilA ($LALF_{32-51}$-E7 + QuilA). By the end of the 32-day observation period, the therapeutic anti-tumor activity induced by $LALF_{32-51}$-E7 alone was comparable to that induced by $LALF_{32-51}$-E7 co-administered with VSSP, OMV or QuilA as adjuvants.

To determine the effect of $LALF_{32-51}$-E7 mixed with different adjuvants on long-term survival, the animals were observed for a period of 110 days. Compared with the $LALF_{32-51}$-E7-treated mice, only the group immunized with $LALF_{32-51}$-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 $LALF_{32-51}$-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 $LALF_{32-51}$-E7 immunization is dose dependent. The effectors cells derived from mice immunized with 60 μg of $LALF_{32-51}$-E7 were significantly more cytotoxic than those from mice immunized with 30 μg of $LALF_{32-51}$-E7. The highest ELISPOT numbers were found in mice receiving $LALF_{32-51}$-E7 alone. The number of IFN-γ-secreting splenocytes in mice immunized with $LALF_{32-51}$-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 ($LALF_{32-51}$-E7 fusion protein) with promissory perspectives to treat HPV16 related malignancies, based on the covalent linkage of a novel CPP with immunostimulatory properties ($LALF_{32-51}$ 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 $LALF_{32-51}$ allows it to enter cells and thereby significantly improves the presentation of E7-derived peptides to T-cells *in vitro*. The $LALF_{32-51}$-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 $LALF_{32-51}$. Furthermore, fusion to $LALF_{32-51}$ enhances the therapeutic anti-tumor activities of viral protein-based vaccine.

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