Targeting and retention of HPV16 E7 to the endoplasmic reticulum enhances immune tumour protection

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Abstract

The endoplasmic reticulum (ER) is where the major histocompatibility complex (MHC) class I molecules are loaded with epitopes to cause an immune cellular response. Most of the protein antigens are degraded in the cytoplasm to amino acids and few epitopes reach the ER. Antigen targeting of this organelle by Calreticulin (CRT) fusion avoids this degradation and enhances the immune response. We constructed a recombinant adenovirus to express the E7 antigen with an ER-targeting signal peptide (SP) plus an ER retention signal (KDEL sequence). In cell-culture experiments we demonstrated that this new E7 antigen, SP-E7-KDEL, targeted the ER. Infection of mice with this recombinant adenovirus that expresses SP-E7-KDEL showed interferon induction and tumour-protection response, similar to that provided by an adenovirus expressing the E7 antigen fused to CRT. This work demonstrated that just by adding a SP and the KDEL sequence, antigens can be targeted and retained in the ER with a consequent enhancement of immune response and tumour protection. These results will have significant clinical applications.

Keywords: KDEL sequence • endoplasmic reticulum • signal peptide • calreticulin • HPV16 • cancer immunotherapy

Introduction

The $CD8^+$ T cells are key players in the immune response to intracellular infections and tumours. Recognition of any intracellular or viral protein by $CD8^+$ cytotoxic T lymphocytes requires an initial cytosolic proteolytic processing by proteasomes to peptide products, which are then translocated by transporters associated with antigen processing (TAP) into the endoplasmic reticulum (ER), where they assemble with major histocompatibility complex (MHC) class I molecules to be presented to the cell surface [1].

Most peptides released from the proteasome into the cytosol are promptly degraded by cytosolic endopetidases and aminopeptidases to single amino acids before they can 'escape' by binding to the TAP and enter the ER. It has been calculated that only one peptide binds to an MHC class I molecule from 10^4 degraded proteins [2]. This explains the relatively subtle efficiency of antigen presentation by class I molecules. Coupled with this, cervical cancer cells (as well as a number of other malignant tumours) often show down regulation or, in a subset of cases, complete loss of MHC class I expression, most likely as a mechanism of immune escape [3].

Previous studies have shown that antigens linked to Calreticulin (CRT), an abundant 46 kD Ca^{2+}-binding protein located in the ER, are directly expressed in the ER, avoiding cytoplasmic degradation [4]. In this way all antigen molecules would be available for degradation into the ER and a larger number of peptides might be generated to be loaded onto MHC-I. With this strategy, peptide-specific $CD8^+$ T-cell responses and antigen-antitumour activity against a murine model of cervical uterine cancer were enhanced by fusion of the human papilloma virus (HPV-16) antigen E7 to CRT

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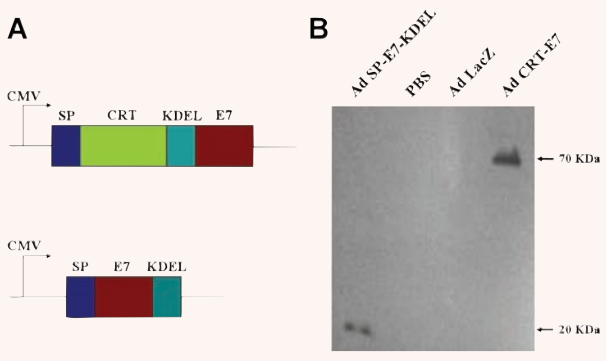

Fig. 1 Generation of an adenovirus expressing SP-E7-KDEL. (**A**) Graphic representation of the gene constructs. Fusion of the HPV16 E7 protein and different signal sequences was made *in silico* and then gen synthesized. (**B**) Detection of the SP-E7-KDEL and CRT-E7 protein expression by Western blot. HEK293 cells were infected with the adenovirus expressing SP-E7-KDEL, CRT-E7, and LacZ at a multiplicity of infection (MOI) of 10. Twenty-four hours after infection cell-protein extracts were prepared and subjected to SDS-PAGE electrophoresis, and transferred to nitrocellulose membranes. These were incubated first with a monoclonal antibody against HPV16 E7 protein, then with the rabbit antimouse IgG-HRP second antibody, and developed with Super Signal West Pico stable peroxide solution.

[4, 5]; however, the mechanism involved remains poorly understood. More significantly, the CRT has been shown to generate the greatest immune response compared to other adjuvant fusions, such as the sorting signal of the lysosome-associated membrane protein, *Mycobacterium tuberculosis* heat-shock protein 70 (HSP70) and the translocation domain of *Pseudomonas aeruginosa* exotoxin A. To simplify this strategy and to determine if most of the antitumour properties conferred by calreticulin are dependent on its ability to be retained in the ER, we engineered a new version of an E7 gene bearing the signals required for ER targeting, signal peptide (SP: MLLPVPLLLGLLGLAAAL) and ER retention (KDEL).

We have previously shown that vaccination with an adenovirus expressing CRT chimerically linked to the HPV-16 E7 antigen causes stronger E7-specific immune responses compared to vaccination with an adenovirus vector expressing only the E7 protein [6].

Herein, we constructed an adenovirus expressing the E7 antigen with a SP at its amino end and a KDEL sequence at its carboxyl end (Fig. 1A) (For details on adenoviral constructs see Supporting Information). After adenovirus production, human embryonic kidney 293 (HEK293) cells were infected with the different adenoviruses (SP-E7-KDEL, CRT-E7 or LacZ), and the protein extracts were analysed by Western blot. As shown in Fig. 1B, the SP-E7-KDEL adenovirus expresses an 18 kD protein, as expected, in contrast to the 70 kD band expressed by the CRT-E7 adenovirus. To demonstrate that the SP-E7-KDEL fusion protein has been

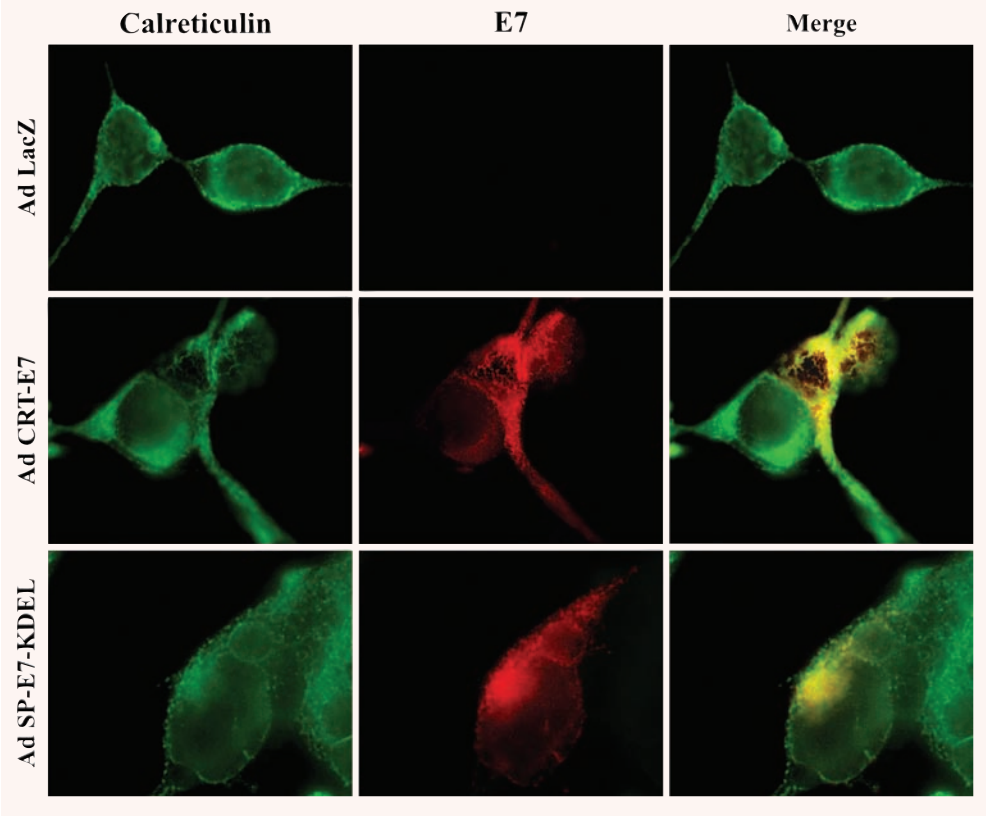

Fig. 2 Distribution of the proteins SP-E7-KDEL and CRT-E7 to the ER. HEK293 cells infected at a MOI of 5 with an adenovirus expressing SP-E7-KDEL, Ad-CRT-E7 or LacZ, were cultured over glass slides for 48 hrs. Cells were fixed and first incubated with rabbit anti-calnexin MAb or mouse anti-E7, and then with AlexaFluor 488-conjugated goat anti-rabbit IgG or AlexaFluor594-conjugated goat antimouse. The glass slides were mounted with antifading medium, Vectashield with DAPI, and covered with cover slips. Samples were examined by using a fluorescence microscope.

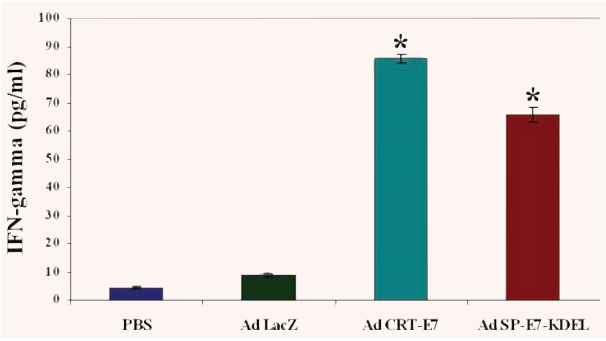

Fig. 3 Detection of IFN-γ induction by ELISA. Groups of three mice were immunized by intraperitoneal (ip) injection with adenovirus expressing SP-E7-KDEL, CRT-E7 or LacZ (5×10^{10} VPs/mouse). Splenocytes from these animals were harvested after 7 days, and then stimulated *in vitro* with the E7 CTL immunodominant epitope RAHYNIVTF (amino acids 49–57) for 3 days. The culture supernatants were collected and levels of IFN-γ were determined using the IFN-γ ELISA kit, according to the manufacturer's protocol. The basal levels of IFN, without peptide stimulation, were substracted. $^*P < 0.05$, compared against controls LacZ and PBS.

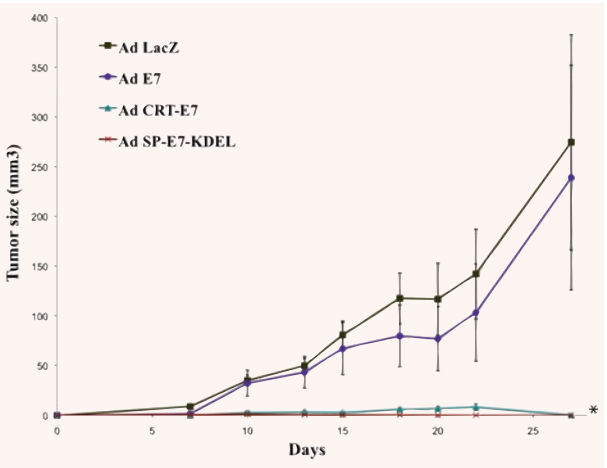

Fig. 4 Tumour protection assay. Groups of five mice were vaccinated by ip injection with 5×10^{10} VPs/mouse of adenovirus expressing SP-E7-KDEL, CRT-E7, E7 or LacZ. One week later, mice were subcutaneously (sc) challenged with 5×10^4 TC-1 cells in the right leg. Tumour growth was monitored twice a week with a calliper. Tumour size was expressed as the mean of two perpendicular diameters. Values and bars represent the mean and Standard Error of tumour size. Ad-CRT-E7 and Ad-SP-E7-KDEL are statistically different from Ad-E7 and Ad-LacZ ($^*P < 0.05$).

distributed to the ER, we did immunofluorescent staining of cells infected with the different adenoviruses using antibodies against E7 protein and calnexin (a well-characterized marker for the ER) [7]. Previous studies in our laboratory and by others have demonstrated that the cytoplasmic/nuclear localization of E7 is lost by fusion to calreticulin which sequesters E7 to the ER [4–6]. As shown here again in Fig. 2, E7 (red signal) colocalization with the calnexin protein (green signal) confirms the distribution of the CRT-E7 to the ER. Similarly, E7 bearing the SP and the ER retention sequence from CRT (SP-E7-KDEL) had the same pattern as the calnexin marker, demonstrating that the signal sequences added to E7 from CRT are able to replace its function of ER retention.

To evaluate if the SP and KDEL signals have the same adjuvant effect as CRT did with E7, groups of female adult C57BL6 mice treated with adenovirus expressing either the SP-E7-KDEL or CRT-E7 were analysed for levels of interferon (IFN)-γ, a signature cytokine for Th1 responses and the effector function of $CD8^+$ T cells, critical for tumour eradication [4–6]. We previously had shown that fusion of CRT to E7 increases, about five times, the release of IFN-γ from splenocytes of mice immunized with an Adenovirus expressing this fusion (50 pg/ml) *versus* an Adenovirus expressing just E7 (10 pg/ml), and increases this eight times *versus* and Adenovirus expressing LacZ (6 pg/ml) [6]. As shown in Fig. 3, we confirmed again that splenocytes from mice immunized with Ad-CRT-E7 generated significant levels of IFN-γ when stimulated *in vitro* by the E7 epitope. The IFN-γ response upon immunization with the adenovirus expressing the SP-E7-KDEL showed also a significant induction of IFN-γ as the Ad-CRT-E7 did (eight times over the control Ad-LacZ).

We then evaluated the protective effect of these chimeric proteins in a murine tumoral model. For this purpose, groups of female adult C57BL6 mice were challenged with HPV-16 E7-expressing murine tumour cells, designated TC-1, 1 week after immunization with adenovirus expressing CRT-E7, SP-E7-KDEL, E7 or LacZ. As seen in Fig. 4, rapid tumour growth was observed in the group of mice immunized with the control adenovirus expressing LacZ, all of whom had to be killed. As expected, immunization with an Adenovirus expressing E7 did no protect the mice against tumour development, which is because of its poor immunogenicity [4–6]. All the mice had to be killed as well. In contrast, all the mice vaccinated with the adenovirus expressing SP-E7-KDEL or CRT-E7 eliminated the tumour. This similar protective effect demonstrates that the SP and KDEL signals are enough to enhance the antitumour immune response.

Taken together, our results indicate that the adjuvant antitumour effect attributed to CRT [8–12] is mainly conferred by its SP and KDEL sequences, which allow antigens to enter and be maintained in the ER. The KDEL sequence is emerging as a significant antitumour immunity sequence. Recently, it was shown that dendritic cells loaded with exogenous peptides bearing the KDEL motif showed a more efficient antitumour activity than dendritic cells pulsed with peptides without KDEL [13]. Even though the mechanism of epitope generation in the ER is poorly understood, the KDEL sequence might result in a longer trafficking time through the ER, increasing the possibility of degradation and consequently, peptide generation and loading by the MHC-1 molecule. It will be worth determining the contribution of the SP itself to the adjuvant effect compared to the addition of both SP and KDEL sequences [14, 15]. Another advantage of this strategy is to

prevent any putative adverse effect caused by overexpression of CRT, because of, for example its role in Ca^{2+} storage and transport into the ER [16]. It has been demonstrated that fusion of CRT also enhances the humoral response of antigens such as the receptor-binding domain (domain IV) of the protective antigen (PA[dIV]) of *Bacillus anthracis* [17] and a SARS coronavirus antigen [18]. Even though the pathway is unknown, it is expected that our strategy might potentiate this response as well. In conclusion targeting of antigens to the ER confers a striking immunogenic potential in gene cancer therapy. This work represents an important contribution for basic research and future clinical translation.

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Supporting Information

Materials and methods

Mice. Female C57BL6 mice (6–8 weeks old) were purchased from Harlan (México City, México), housed in the animal facilities of the Histology Department of the School of Medicine, UANL, and cared for in conformity with good laboratory practice guidelines.

Cell lines. HEK293 cells were maintained in advanced DMEM supplemented with 4% heat-inactivated foetal bovine serum (FBS). TC-1 cells derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6 and E7 and the c-Ha-ras oncogenes, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. All of the cell lines were purchased from American Type Culture Collection (Manassas, VA, USA).

Adenovirus. Fusion of the HPV16 E7 protein and different signal sequences was done *in silico* and then synthesized by the GenArt Company. Genes were subcloned in pShuttle vector and then recombined with the adenoviral genome. Replication-deficient E1 = E3-deleted adenovirus containing the gene of interest was amplified in 293 cells. Infected cells were harvested and lysed, and the viral particles (VPs) were purified using the ViraBind Adenovirus Purification kit (Cell Biolabs, Inc., San Diego, CA, USA).

Western blot. HEK293 (2×10^5) cells were plated in 12-well plates and infected the next day with the different adenoviruses to be tested (MOI 10). Twenty-four hours after infection cells were lysed with ProteoJet Mammalian Lysis Solution (Fermentas). Lysed cells, mixed with 30 μl 2× SDS loading buffer, were heated at 100°C for 5 min., and centrifuged at 13,000 rpm for 10 min. Protein samples were separated by electrophoresis gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 hr with 10% non-fat dry milk in TBST (135 mM NaCl, 2.7 mM KCl, 24.8 mM TRIS·HCl, 0.05% Tween 20, pH 7.4) and then probed with an antibody in TBST for 1 hr at 25 °C. A monoclonal antibody against HPV16 E7 protein (Zymed) was used to probe the expression of E7. Blots were rinsed with TBST, incubated with rabbit antimouse IgG-HRP second antibody (Sigma) for 1 hr, then rinsed with TBST again, and developed with Super Signal West Pico stable peroxide solution (Pierce).

Immunofluorescence. HEK293 cells infected at a MOI of 5 with Ad-LacZ, Ad-CRT-E7 or Ad-SP-E7-KDEL were cultured over glass slides for 48 hrs. Cells were washed in PBS, and fixed with a methanol:acetone solution for 10 min at –20 °C; blocked with a 2% BSA in PBS solution, and then incubated with rabbit anti-calnexin MAb (Santa Cruz Bio-technology) or mouse anti-E7 (Zymed) at a concentration of 1 μg/ml for 1 hr at room temperature. After several washes with PBS, cells were incubated with AlexaFluor 488-conjugated goat anti-rabbit IgG or AlexaFluor 594-conjugated goat antimouse (Invitrogen) at a concentration of 10 μg/ml for 1 hr. The slides were then washed with PBS containing 1% BSA and mounted with antifading medium, Vectashield with DAPI (Vector), and covered with cover slips. Samples were examined by using a Leica fluorescence microscope.

Detection of IFN-γ. Groups of three mice were immunized by intraperitoneal (ip) injection with adenovirus expressing SP-E7-KDEL, CRT-E7 or LacZ (5×10^{10} VPs/mouse). Splenocytes from these animals were harvested after 7 days and were stimulated with E7 CTL immunodominant epitope RAHYNIVTF (amino acids 49–57) in 24-well plates for 3 days. Culture supernatants were collected and levels of IFN-γ were determined using an IFN-γ ELISA kit (R&D) according to the manufacturer's protocol. Data are representative of two experiments.

Tumour protection assay. Groups of five mice were vaccinated by ip injection with 5×10^{10} VPs/mouse of adenovirus expressing SP-E7-KDEL, CRT-E7 or LacZ. One week later, mice were subcutaneously (sc) challenged with 5×10^4 TC-1 cells in the right leg. Tumour growth was monitored twice a week with a calliper. The tumour size was expressed as the mean of two perpendicular diameters. Values and bars represent the mean and standard error of tumour size. Animals bearing tumours were killed at day 28. Data are representative of two experiments.

Statistical analysis. Statistical analysis was done using an ANOVA test. *P*-values < 0.05 were considered significant.

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