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 downregulation 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 cytosplasmic 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
[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: MLLPVLGLLLGLAAAL) 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

![Calreticulin, E7, Merge](image)
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-\(\gamma\)) by ELISA. Groups of three mice were vaccinated by intraperitoneal (ip) injection with adenovirus expressing SP-E7-KDEL, CRT-E7 or LacZ (5 \(\times\) 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-\(\gamma\) were determined using the IFN-\(\gamma\) ELISA kit, according to the manufacturer’s protocol. The basal levels of IFN, without peptide stimulation, were subtracted. *\(P < 0.05\), compared against controls LacZ and PBS.

To evaluate if the SP and KDEL signals have the same adjuvant effect as CRT did with E7, groups of female adult C57BL6 mice were vaccinated by ip injection with 5 \(\times\) 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 \(\times\) 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 (*\(P < 0.05\)).
Lysed cells, mixed with 30 lysed with ProteoJet Mammalian Lysis Solution (Fermentas) be tested (MOI 10). Twenty-four hours after infection cells were plates and infected the next day with the different adenoviruses to amplified in 293 cells. Infected cells were harvested and lysed, and from CONACYT of Mexico. Thanks to Dr. Ellis Glazier for editing this English language text.

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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 onco-genes, 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 ViCap 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-cal-nexin 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 caliper. 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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