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Highly protective E2–CSFV vaccine candidate produced in the mammary gland of adenoviral transduced goats

Jorge R. Toledo [a,*], Oliberto Sánchez [a], Raquel Montesino [b], Omar Farnos [a], Maria P. Rodríguez [a], Pastor Alfonso [c], Nayrobis Oramas [a], Elsa Rodríguez [b], Elaine Santana [a], Ernesto Vega [c], Llilianne Ganges [d], Maria T. Frias [c], José Cremata [b], Maritza Barrera [c,1]

[a] *Animal Biotechnology Department, Centre for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba*
[b] *Chemistry-Physic Department, Centre for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba*
[c] *National Center for Animal and Plant Health (CENSA), Apdo 10, San José de Las Lajas, Havana, Cuba*
[d] *Dpto. Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), 28040 Madrid, Spain*

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Abstract

Classical swine fever virus is the etiological agent of the most economically important highly contagious disease of swine worldwide. E2 is the major envelope glycoprotein present as a homodimer on the outer surface of the virus and represents an important target for the induction of neutralizing immune response against the viral infection. The E2 extracellular domain was expressed in the milk of adenoviral transduced goats at the highest level about 1.2 g/L. The recombinant glycoprotein was purified from clarified serum milk by a single metal chelate affinity chromatography step, as a homodimer of approximately 100 kDa and purity over 98%. Glycosylation analysis showed the presence of oligomannoside, hybrid and complex type *N*-glycans, attached to the recombinant E2.

The capacity of goat milk-derived E2 antigen to protect pigs from both classical swine fever clinical signs and viral infection was assessed in a vaccination and challenge trial. The immunized pigs became protected after challenge with 10^5 LD_{50} of a highly pathogenic CSFV strain. In the context of veterinary vaccines, this expression system has the advantages that the recombinant antigen could be harvested in about 48 h after adenoviral transduction with expression levels in the range of g/L. This approach may turn into a scalable expression system for the assessment and production of veterinary vaccines.

Keywords: Mammary gland; Adenoviral transduction; Veterinary vaccine; CSFV; Goat milk; E2 glycoprotein

1. Introduction

Several biopharmaceutical proteins require post-translational modifications to display biological activity and stability, which are not performed correctly in non-mammalian cells. Livestock with mammary gland-targeted expression seems to be able to produce valuable recombinant proteins at very low cost in comparison with mammalian cell bioreactors (Clark, 1998; Houdebine, 2000; Niemann et al., 2005). This technology had been focused in the production of human proteins of therapeutic interest like hormones, blood proteins and antibodies (Niemann and Kues, 2003)'. However, as far as we know there are no reports about the production of recombinant antigens in the milk of livestock.

The adenoviral transduction of mammary gland had been developed as a simple and fast method for the production at high levels of recombinant proteins in the milk of ruminants (Sanchez et al., 2004; Toledo et al., 2006; Han et al., 2006). The present issue is regarding to the expression of E2 glycoprotein in the milk of goats.

E2 is the major envelope glycoprotein exposed as a homodimer on the outer surface of the classical swine fever virus

* Corresponding author at: Animal Biotechnology Department, Centre for Genetic Engineering and Biotechnology, Ave. 31 and 158, Cubanacan, Playa, PO Box 6162, Havana 10600, Cuba. Tel.: +53 2716022x5108; fax: +53 7 33 1779.
E-mail addresses: jorge.toledo@cigb.edu.cu (J.R. Toledo), maritza@censa.edu.cu (M. Barrera).
[1] Tel.: +53 4763014x33; fax: +53 64 98104.

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(CSFV) and represents the most significant target for the induction of the immune response in pigs (Rumenapf et al., 1991; Dong and Chen, 2007). The native E2 glycoprotein has a complex tertiary structure with four intrachain disulphide bonds, six potential *N*-glycosylation sites and several interchain disulphide bonds stabilizing the homodimeric and conformational antigenic structure (van Rijn et al., 1994; Ganges et al., 2005; Risatti et al., 2005).

In this work, the E2–CSFV extracellular domain was expressed in the milk of adenoviral transduced goats and it was obtained from clarified serum milk with a high purity level, after a single metal chelate affinity chromatography step. Pigs immunized with this recombinant antigen were fully protected after a challenge with 10^5 PLD_{50} of the homologous highly pathogenic CSFV "Margarita" strain. These results suggest that the E2his recombinant glycoprotein produced in the milk of goats may constitute a vaccine candidate suitable against CSFV.

2. Materials and methods

2.1. Cells and viruses

Human embryonic kidney (HEK-293) cell line (ATTC CRL-1573) was used to generate, propagate and titrate the recombinant adenoviral vectors from human serotype five adenoviruses (Ad5) (Graham et al., 1977). Pig kidney (PK-15) cell line (ATCC CCL-33) was used for E2–CSFV expression in adenoviral transduction assays. Measurement of classical swine fever virus–specific neutralizing antibodies (CSFV–SNA) by neutralizing peroxidase-linked assay (NPLA) (Wensvoort et al., 1986) and CSFV isolation were also conducted in this cell line.

The highly pathogenic CSFV "Margarita" strain used in this study was isolated in Havana in 1958 and has been used since 1965 for vaccine potency tests in Cuba. This virus was grouped, by sequence analyses, within the CSFV genotype 1.2 (Diaz de Arce et al., 1999; Diaz de Arce et al., 2005).

2.2. Adenoviral vector

The replication defective adenoviral vector (Ad–E2his) was previously generated (Sanchez et al., 2007) using the AdEasy adenoviral vector system (He et al., 1998). The Ad–E2his vector contain the cDNA sequence from nt 4 to nt 1092 [AJ704817] of the E2–CSFV extracellular domain (Ganges et al., 2005). The E2 sequence was headed by the tissue plasminogen signal peptide and a hexa-histidine tag was added in the C-terminus (E2his). The Ad–E2his was amplified by HEK-293 cell line infection in two Cell Factory stack chambers (Nunc, Denmark). It was purified from infected cells by three freeze/thaw steps (−20 °C/37 °C) and further centrifugation at 4000 rpm during 30 min. The supernatant was tittered and stored at −70 °C.

2.3. Pig kidney cells-derived E2his obtaining

The PK–E2his antigen was expressed and purified from culture medium of PK-15 cells transduced with the Ad–E2his adenoviral vector, as was previously described (Sanchez et al., 2007).

2.4. Goat mammary gland E2his expression

Mammary adenoviral transduction was carried out as was previously reported (Toledo et al., 2006). Five healthy Saanen goats, 1 year old, were subjected to a 2-week hormonal regime to induce mammogenesis and lactation (Cammuso et al., 2000). The Ad–E2his was infused 25 days later in each mammary gland at a concentration of 5×10^9 gene transfer units (GTU)/mL in phosphate saline buffer (PBS) containing EGTA 36 mM, pH 7.4. One goat was infused with other non-related adenoviral vector (Ad-GFP) and it was taken as placebo. Twenty-four hours after transduction the adenoviral inoculums were thrown out and the milking began 24 h later. All goats were daily milked for the next 20 days.

2.5. Purification of E2his from goat milk

2.5.1. Serum milk clarification

E2his was purified from goat milk corresponding to milking days 1–7. The milk was diluted four-folds in a milk separating buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0) and chilled on ice during 30 min. The mix was separated by centrifugation at 10,000 rpm for 30 min at 4 °C; the fatty layer was discarded and the serum milk was separated from the casein precipitate. Two filtration steps were carried out in a filtration apparatus using 0.8 and 0.4 μm membranes (Sartorius, Germany). The clarified serum milk and the casein precipitate were both analyzed for E2his detection.

2.5.2. Metal chelate affinity chromatography

1 M Tris-HCl, 10 mM imidazole pH 7.2 buffer was added in a relation of 1/10 volume to the clarified serum milk for pH adjustment. It was applied onto a Ni-NTA Agarose column (16 mm × 50 mm, Qiagen, Germany) previously equilibrated in 100 mM Tris-HCl, 1 mM imidazole pH 7.2, at a flow rate of 0.2 mL/min. After serum milk loading, two washing steps were carried out with equilibrium buffer containing imidazole 20 and 50 mM. The E2his protein was eluted with the same buffer containing 200 mM imidazole. Protein purification was monitored by UV absorption at A_{280nm}. The eluted fraction was dialyzed against 10 mM NaH_2PO_4 pH 7.4.

2.6. E2his inmmunoidentification

E2his samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions (1% glycerol, 0.4% SDS, 0.1% β-mercaptoethanol, 12.5 mM Tris-HCl, pH 6.6) or non-reducing conditions (1% glycerol, 0.4% SDS, 12.5 mM Tris-HCl, pH 6.6). The separated samples were transferred onto nitrocellulose membrane (Millipore, USA) and was subjected to Western-blotting analysis with 1G6 anti-E2–CSFV monoclonal antibody (mAb) as primary and goat anti-mouse-horseradish peroxidase conjugate IgG-Fc (SIGMA, USA) as second antibody. The hybridoma for 1G6 mAb was kindly donated by Dr. Rumenaft (Giessen University, Germany).