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Adenovirus based HPV L2 vaccine induces broad cross-reactive humoral immune responses

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ABSTRACT

Oncogenic high-risk human papillomavirus (HPV) infections cause a substantial number of genital and non-genital cancers worldwide. Approximately 70% of all cervical cancers are caused by the high-risk HPV16 and 18 types. The remaining 30% can be attributed to twelve other high-risk HPV-types. Highly efficacious 2-valent, 4-valent and 9-valent L1 protein based prophylactic HPV vaccines are available however with limited cross-protection. To further increase the coverage, development of a multivalent cross-protective HPV vaccine is currently focused on the conserved N-terminus of HPV's L2 protein. We have developed a vaccine candidate based on the rare human adenovirus type 35 (HAdV35) vector that displays a concatemer of L2 protein epitopes from four different HPV-types via protein IX (pIX). A mix of two heterologous HAdV35 pIX-L2 display vectors present highly conserved linear epitopes of nine HPV-types. Each HAdV35 pIX-L2 display vector exhibits a good manufacturability profile. HAdV35 pIX-L2 display vaccine vectors were immunogenic and induced neutralizing antibodies against HPV-types included in the vaccine and cross-neutralizing antibodies against distant a HPV-type not included in the vaccine in mice. The HAdV35 pIX-L2 display vectors offer an opportunity for a multivalent HAdV-based prophylactic HPV vaccine.

1. Introduction

Oncogenic high-risk human papillomavirus (HPV) infections are responsible for almost 5% of all cancers worldwide, with substantially higher rates in developing countries [1,2]. Twelve high-risk HPV genotypes are implicated as the causative agents of anogenital and oropharyngeal cancers in woman and men [1,3]. Approximately 70% of the cervical cancer cases worldwide are caused by the high-risk HPV16 and 18 genotypes. The remaining 30% of cervical cancer cases are caused by other high-risk types: HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and probably HPV68 [2,4]. Low-risk HPV genotypes such as HPV6 and 11 can cause benign but debilitating genital and cutaneous warts [1,2]. Three L1 virus-like particles (VLP) based prophylactic HPV vaccines are currently available: 2-valent (HPV16 & 18) *Cervarix* (GSK), 4-valent *Gardasil* (HPV6, 11, 16 and 18) and 9-valent *Gardasil*9 (HPV6, 11, 16, 18, 31, 33, 45, 52 and 58) (Merck) [5–7].

Although L1 VLP vaccines have proven to be highly efficacious, due to limited cross-protection wide HPV coverage can only be achieved by addition of L1 component of each HPV genotype [8] increasing the vaccine complexity. Therefore, there is a need for an alternative less complex prophylactic HPV vaccine with broad HPV coverage [9,10], and efforts have been invested in generating an HPV vaccine based on the other HPV capsid component, the L2 protein [9,10]. L2 protein-based vaccine might have potential for broad HPV coverage with a single vaccine due to the highly conserved cross-protective linear neutralizing antibody (nAb) epitopes in the L2 protein N-terminus [10,11]. Immunization with adjuvanted N-terminus L2 peptide concatemers (i.e. repeats) induced cross-protective antibody responses in different animal models [12–16]. Surface exposed repetitive presentation of the L2 epitope is believed to improve the L2-specific cross-protection as demonstrated by some antigen-display platforms [17]. For instance, the

Abbreviations: a.a, amino acid; AdV, adenoviral; HAdV35, human adenovirus 35; HPV, human papillomavirus; MSD, Meso Scale Discovery; nAb, neutralizing antibody; pIX, protein IX; VLP, virus-like particle; VNA, virus neutralization assay; VP, viral particles; VPN, viral passage number.

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adjuvanted scaffold L2 epitope presentation induced L2-specific antibodies in mice [18]. Similarly, several different experimental adjuvanted VLP-based L2-display vaccine designs induced L2-specific broad and cross-reactive antibodies in animal models: HPV L1-based VLP [19,20], bacteriophage VLP [21–23], adeno-associated virus particles capsid display [24] and human adenovirus 5 (HAdV5) L2 hexon-display [25].

Replication-incompetent AdV vectors are attractive as vaccine vectors due to their clinically acceptable safety profile, potent antigen-specific immune responses and good manufacturability [26]. Vaccination with AdV vectors against infectious diseases such as HIV and Ebola has proven promising in clinical trials without an adjuvant [27–30]. Additionally, AdV vectors can be modified to display antigens via capsid proteins such as hexon and pIX [31]. AdV pIX-display vectors induce potent immune responses against *Yersinia pestis* and malaria in animal models [32–34]. We hypothesized that with the adenoviral (AdV) protein IX (pIX)-display vaccine platform we could generate a multivalent L2 protein display prophylactic HPV vaccine.

To evaluate the feasibility of a multivalent replication-incompetent AdV pIX-display based prophylactic HPV vaccine, HAdV35 vectors displaying L2-epitope concatemers fused to the pIX C-terminus (pIX-L2) were generated and extensively characterized. Unlike HAdV5 vectors [35,36], vectors derived from rare HAdV35 and HAdV26 types [36–39] are less likely to be hampered by high levels of pre-existing immunity. HAd35 pIX-L2 vectors showed good manufacturability that was comparable to the non-modified control vector, with consistent batch-to-batch, viral titer yields, genetic stability and stability after 1 year incubation at cold-chain relevant 2–8 °C temperatures [40,41]. Each single HAdV35 pIX-L2 vector displaying different HPV L2 concatemers elicited humoral immune responses in mice, against the HPV types included and not included in the vaccine, which were further enhanced by mixing two heterologous HAdV35 pIX-L2 vectors in one vaccine formulation. These findings indicate that the pIX-L2 display vector offers an opportunity for an AdV-based multivalent prophylactic HPV vaccine.

2. Methods

2.1. Vector generation

The replication-incompetent E1/E3-deleted HAdV35 vectors displaying HPV L2 protein concatemers (synthesized and codon-optimized [*Homo sapiens]* by GeneArt]ThermoFisher Scientific]) via pIX were generated in E1-complementing PER.C6® cells, CsCL-purified and characterized as previously described [34].

2.2. pIX capsid incorporation

Capsid incorporation of pIX-L2 proteins was determined by western blot with reduced/denatured CsCL-purified viral particles (VP) as previously described [34]. ELISA was performed by immobilizing CsCL-purified intact VP (10^{10} VP/well) as previously described [42].

2.3. HAdV proteome analysis by RP-UPLC and LC-MS/MS

Reversed-Phase-Ultra-Performance-Liquid-Chromatography (RP-UPLC) on an ACQUITY BEH C4 Column, 300 Å, 1.7 μm, 2.1 mm × 150 mm with ACN + 0.17% TFA gradient was performed to evaluate the protein content of the purified HAdV35 vectors (280 nm absorption). pIX/pIX-L2 peak area abundance (%) was determined relative to the peak area sum of all the viral proteins.

Protein sequences of pIX-L2 were determined by Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis. pIX/pIX-L2 bands were isolated from purified HAdV35 vector, separated on a 4–12% Bis-Tris gel SDS-PAGE gel (Thermo Fisher Scientific), stained with SilverQuest™ and treated with Trypsin-V5111 (Promega) according to manufacturer's recommendations. The digested proteins were separated on a 150 × 2.1 mm reversed-phase C18 BEH300 UPLC column with 2–50% ACN + 0.1% FA gradient connected to mass spectrometer Waters Synapt G2 ESI-Q-TOF. Peptide sequence analysis was performed using BiopharmaLynx version 1.3.2 (Waters).

2.4. Viral growth kinetics and genetic stability in producer cell line

HAdV35 vectors growth kinetics in the suspension PER.C6® producer cell line were evaluated by analyzing the VP/mL titers with CMV-promoter-specific quantitative PCR (QPCR) (0–4 days post infection). The cells were lysed with 1% Triton™ X-100 (Sigma-Aldrich) and treated with DNaseI (Roche). TaqMan Gene expression master mix (Life Technologies), 1000x diluted sample and 25 mM $MgCl_2$ with 10 pmol of each primer were used. In the reaction, DNA was denatured at 95 °C for 8 min, followed by 10 s at 95 °C and 30 s at 60 °C for 35 cycles in the QPCR machine (Applied Biosystems Viia7). Genetic stability of the pIX-L2 modification in the HAdV genome during vector production in PER.C6® cells was evaluated at the fourth viral passage number (VPN) beyond the commercial process stage as described by Vogels et al. [43].

2.5. Incubation at 2–8 °C and the infectivity assessment by QPA

HAdV35 vectors were incubated for up to 1 year at 2–8 °C under controlled conditions. HAdV vectors (n = 3) were diluted in formulation buffer (10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 75 mM NaCl, 5% sucrose, 0.02% PS-80, 0.1 mM EDTA, 10 mM Histidine, 0.5% ETOH) to 1×10^{11} VP/mL in a glass 1 mL vial. Infectivity (IU/mL) testing by Quantitative-PCR-based Potency Assay (QPA) was performed as described using CMV primers/probe [44]. Change from baseline values ($\Delta\log_{10}$ values) were calculated by subtracting the log10 value at time point 0 from the log10-transformed values at later time points.

2.6. Mice immunization and ethical statement

Six-to-eight-week-old female CB6F1 mice (Charles River) were vaccinated intramuscularly two times at an 8-week interval with HAdV-vectors in the quadriceps of both hind legs with 1×10^8 or 1×10^{10} VP/mouse of each HAdV35 vector type (e.g. total 2×10^8 or 2×10^{10} VP). As a positive control for the L2 pseudovirions virus neutralization assay (VNA), mice were vaccinated three times at 4-week interval with 1/10 of a full-human dose of 4-Gardasil (Merck). Animal handling was performed according to the Dutch Animal Experimentation Act and Directive 86/609 of the Council of the European Committee after approval by Janssen Dier Experimenten Commissie (permit number 21300).

2.7. L2-specific-antibody in mice by MSD-ELISA

L2 HPV-type-specific serum IgG antibody responses were determined by using the Meso Scale Discovery (MSD) multivalent immunoassay. Each plate contained ten spots per well covered with one of HPV L2 (10–38 amino acids [a.a].) antigen peptides of HPV6, 11, 16, 18, 31, 33, 45, 52(58), 59 or BSA protein control. Mouse serum samples were analyzed in the assay according to manufacturer's recommendations and read on a MSD SectorS-600-reader. Antibody titers-$\log_{10}$ were calculated using R3.1.1 software (R Core Team 2013).

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