Bioorganic & Medicinal Chemistry Letters xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Combined synthetic and recombinant techniques for the development of lipoprotein-based, self-adjuvanting vaccines targeting human papillomavirus type-16 associated tumors

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ARTICLE INFO

Article history:
Received 25 September 2015
Revised 14 October 2015
Accepted 16 October 2015
Available online xxxx

Keywords:
Cancer
Human papillomavirus
Pam2Cys
Protein engineering
Vaccine

ABSTRACT

Human papillomaviruses (HPVs) are associated with various cancers, with HPV16 linked to more than half of cervical cancer cases. Vaccines to prevent HPV infection and cancer development have proven effective, but are not useful in individuals with prior HPV exposure. Treatment vaccines to eradicate or control HPV-associated lesions are therefore desirable for these patients. Herein we describe the development of a process to enable the production of semisynthetic vaccines based on the site-specific attachment of synthetic bacterial lipid analogs (e.g., Pam2Cys) to a non-oncogenic mutant HPV16 E7 protein to generate molecularly defined vaccines. Many cytotoxic lymphocyte (CTL) epitopes from E7 are delivered by this approach; potentially ensuring that large numbers of immunized individuals can generate CTLs to clear HPV infected cells. Delivery of this construct reduced the growth of HPV16-associated tumors in a TC1 mouse model, the effects of which were better than the potent CTL epitope HPV16 E7(44–57) administered with Montanide ISA51 adjuvant.

Human papillomavirus (HPV) is the causative agent for cervical cancer, the third most common female cancer, and various other cancers (e.g., certain head and neck tumors).[1] This association has inspired research into vaccines to prevent HPV infection, culminating in the commercialization of L1-based virus-like particles (VLPs; e.g., Gardasil, Merck & Co and Cervarix, GSK). Despite their success, these vaccines are ineffective in individuals who have had prior HPV infection.[1] Vaccines to eradicate or control HPV-associated lesions are therefore of interest, with therapeutic vaccines that enhance T cell-mediated killing of HPV infected cells (expressing E6 and E7 HPV oncogenes) the subject of human trials (reviewed in Refs. [1] and [2c]).

Many groups[1,2] have investigated therapeutic HPV peptide vaccines, with most focused on E7-derived peptides from HPV type 16 (HPV16), which causes over 50% of cervical cancers.[1] Several cytotoxic lymphocyte (CTL) epitopes have been mapped from the E7 protein (see Fig. 1A; CTL epitopes marked with a line over the sequence),[3] which have controlled or eradicated HPV16-associated tumors in mouse[2a,b] and human trials.[4] Using these epitopes, simple, chemically defined synthetic vaccines can be produced.[1] The simplification of proteins down to defined peptide antigens however reduces the vaccine CTL epitope mix, limiting efficacy to individuals expressing human leukocyte antigen (HLA) molecules that bind these epitopes. The utility of such vaccines could therefore be improved by increasing the number of CTL epitopes. Methods to achieve this include mixing overlapping peptides covering the entire HPV16 E6 and E7 sequences (known as 'synthetic long peptides'),[5] or selected epitopes from these proteins.[4] These approaches however greatly increase vaccination costs due to the need to synthesize and purify multiple peptides.

Recombinant expression of engineered proteins provides one means to overcome these difficulties. Full-length or truncated E6 and E7 proteins, and E6–E7 fusions,[1] that have been mutated to remove their oncogenic potential, have the capacity to be overexpressed at lower cost compared with synthetic approaches incorporating multiple peptide antigens, and include significantly

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http://dx.doi.org/10.1016/j.bmcl.2015.10.049
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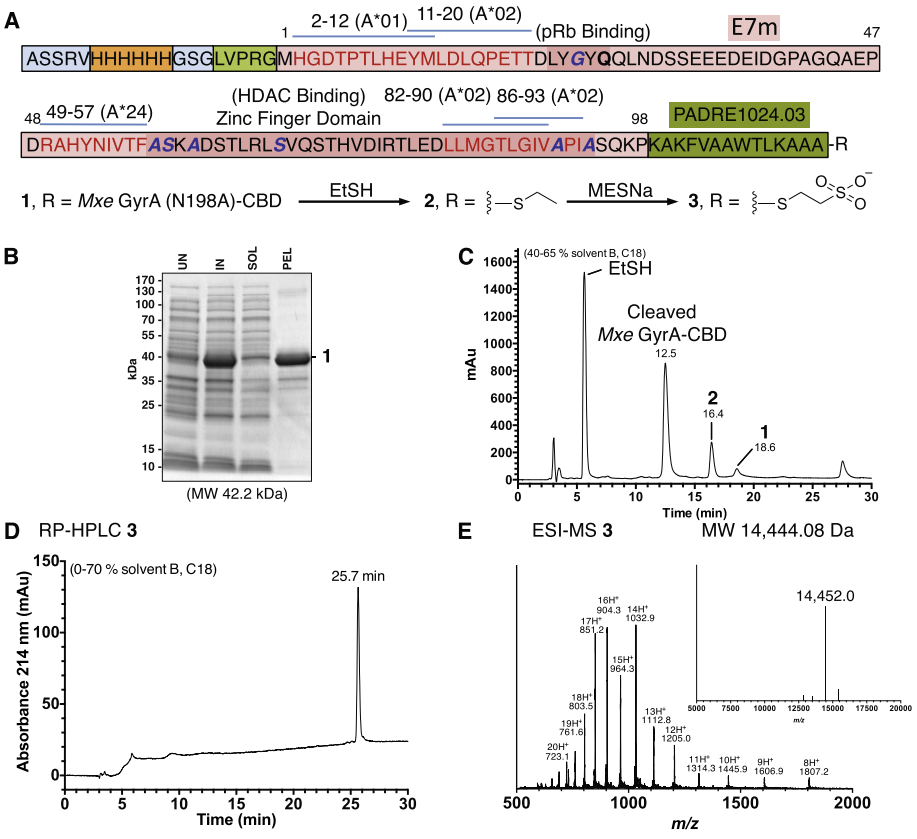

Figure 1. Inactivated HPV16 E7 (E7m) proteins **1**–**3**. (A) E7m sequence; CTL epitopes (line over) and HLA restriction; inactivating (bold) and cysteine mutations (italics); and conversion of intein fusion protein **1** to thioester proteins **2** and **3**. (B) SDS–PAGE of **1** protein expression (whole cell uninduced (UN) and induced (IN) bacteria; soluble cytoplasmic (SOL) and inclusion body pellet (PEL)). (C) RP-HPLC monitoring of *Mxe* GyrA intein-CBD cleavage from **1** with EtSH to yield **2**. (D) Analytical RP-HPLC and (E) electrospray ionization-mass spectrometry (ESI-MS) data (deconvoluted spectra inset) for pure E7m thioester protein **3**.

more CTL epitopes. The low immunogenicity of these proteins however requires their delivery with adjuvants,[1a] of which there are very few known to be capable of stimulating potent CTL responses in humans without being overly toxic.

One promising adjuvant approach for eliciting CTL responses against protein antigens involves the covalent attachment of synthetic or bacterial lipopeptide adjuvants containing dipalmitoyl-(Pam2Cys) or tripalmitoyl-*S*-glycerol cysteine (Pam3Cys) onto peptide antigens.[6] To extend the utility of this approach, we have optimized techniques to enable the site-specific, and efficient conjugation of synthetic bacterial lipopeptide adjuvants containing Pam2Cys or Pam3Cys, or engineered lipid adjuvants (lipid core peptide; LCP) onto engineered recombinant proteins featuring multiple end-to-end linked peptide antigens (termed a 'polytope').[7] These techniques produce a single, chemically defined product, with an inbuilt adjuvant that stimulates potent humoral immune responses. Herein, we aim to extend this strategy toward cellular immune responses (e.g., CTL and helper T cell) to develop therapeutic anti-tumor vaccines. For this purpose a previously reported recombinant E7 protein, which has been mutated to remove its transforming activity (E7m;[8] mutations indicated in Fig. 1A) was selected as an antigen, and modified (**1**–**3**; Fig. 1A) to enable the site-specific attachment of a synthetic lipopeptide adjuvant containing Pam2Cys (**4**; Figs. 2A, B and S1). Using this construct (**5**; Fig. 2A), we aimed to compare the capacity of this lipoprotein vaccine to reduce the growth HPV16-associated tumors with approaches based on the potent HPV16 $E7_{49–57}$[3c] CTL epitope including: (i) a Pam2Cys fusion (**6**; Fig. 3A), which also incorporates the $E6_{43–57}$ CTL epitope from HPV16 (synthesis under review);[9] (ii) an admix with Montanide ISA 51 (**7**/ISA51; Fig. 3B); and (iii) a previously reported star-polymer conjugate (S4-8Qmin; **8**; Fig. 3C).[2b] In support of our approach, a technique enabling the N-terminal incorporation of a mixture of different tri-acylated bacterial lipids during recombinant protein expression in *Escherichia coli* (*E. coli*) has been applied to HPV16 E7, and demonstrated to shrink tumors in a murine cervical cancer model.[8] This approach however generates a mixture of products, compared to the favorable single, molecularly defined product yielded by the semisynthetic method described herein.

In addition to the previously mentioned E7 mutations, which eliminate E7 oncogenic activity by preventing retinoblastoma protein (pRb) and histone deacetylase (HDAC) binding,[8] the cysteine residues in E7m have also been mutated to prevent the formation of undesirable disulfide-linked species.[8] These mutations preserve most of the CTL epitopes described in the literature[3] (Fig. 1A), with the exception of the $E7_{86–93}$ epitope, which contains a single cysteine to alanine mutation (C91A). Based on binding studies,[3c] E7m CTL epitopes can bind HLA-A*02:01, which is found in roughly 50% of Caucasians,[1b] as well as A*24:01, and A*01:01,[3c] with other unidentified epitopes likely. The variety of E7m CTL epitopes, which are capable of binding diverse HLA molecules, suggests that E7m may provide therapeutic activity in more individuals than approaches based on a single, or few peptide epitopes.

To improve E7m protein expression, the E7m gene was codon optimized for B strain *E. coli* using DNAworks v3.2.2 (http://helixweb.nih.gov/dnaworks/) with a codon frequency table from the codon usage database (http://www.kazusa.or.jp/codon/). This gene was fused to: (i) a promiscuous T helper epitope (PADRE1024.03)[10] to help increase the potency of CTL responses against HPV16 E7;[11]