Advances in peptide-based human papillomavirus therapeutic vaccines

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Abstract

Cervical cancer is the second leading cause of cancer in women worldwide. Human papillomavirus (HPV) is responsible for all cases of cervical cancer. Commercial prophylactic HPV vaccines are now available, but unfortunately these vaccines have no therapeutic effect against established HPV infections. In order to accelerate the control of cervical cancer and treat established HPV infections, it is necessary to develop therapeutic vaccines to eradicate HPV by generating cell-mediated immunity against HPV infected cells. Two HPV-encoded early proteins, the E6 and E7 oncoproteins, are the preferred targets because they are consistently expressed in virtually all cervical cancer cells and are necessary for the induction and maintenance of HPV-associated disease. A variety of vaccine strategies have been employed targeting immune responses to these proteins. Peptide-based vaccines are a promising strategy for the development of therapeutic HPV vaccines because of their safety, stability, and ease of production. This review summarizes the prospects of peptide-based vaccines for the treatment of established HPV infections. We address the challenges that scientists currently face for developing peptide-based vaccines and explore feasible strategies for improving the potency of the induced immune response with the aim of treating established HPV infections.
Introduction

Every year cancer of the cervix (cervical cancer) is diagnosed in about 500,000 women, causing approximately 250,000 deaths annually [1, 2]. The high-risk types of human papillomavirus (HPV) are associated with over 80% of cervical cancers through epidemiological and experimental studies [3]. Two high-risk types, HPV type 16 (HPV-16) and HPV type 18 (HPV-18), are responsible for up to 50% and 20% of all cervical cancers, respectively [4]. The traditional detection of cervical pre-cancers by cervical cytological screening using the Papanicolaou (Pap) smear test has dramatically reduced the incidence of cervical cancer in the developed world due to early intervention with therapy. However, underdeveloped countries are unable to implement comprehensive screening-based programs, so early detection and treatment of cervical pre-cancers is very limited [5, 6]. Patients who are diagnosed with early-stage disease can be treated with surgery, radiotherapy, or chemotherapy, but many patients still succumb to the disease [6, 7]. It is therefore necessary to develop more effective therapies for the treatment of cervical cancer.

Immunotherapy is a promising strategy for the treatment of established HPV infection and cervical cancer. The newly licensed prophylactic HPV vaccines, Gardasil® and Cervarix®, use HPV virus-like particles (VLPs) to generate neutralizing antibodies against the L1 (major) capsid protein. These vaccines are predicted to decrease the morbidity of cervical cancers by approximately 70% [8-11]. Despite the successful prophylactic effects of these vaccines, the high cost, cold storage requirement, and availability of the existing prophylactic HPV vaccines are important limitations to the widespread delivery of these vaccines in developing countries. Thus, the prevalence and associated morbidity of cervical cancer may not be significantly reduced worldwide [12-14]. Prophylactic HPV vaccines that target the L1 capsid have no therapeutic effect on established HPV infection. This is because HPV-infected basal epithelial cells and cervical cancer cells do not express detectable levels of L1 capsid antigen. The time between virus infection and tumor development is typically 10-20 years, so a large proportion of the global population is already infected and cannot be treated by the prophylactic vaccine [11, 12, 15, 16]. To combat established infections, it is necessary to develop a therapeutic vaccine against high-risk HPV, particularly HPV-16 and HPV-18 strains [17-19].

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The foremost aim when developing a therapeutic HPV vaccine is the treatment of existing HPV infections. Many therapeutic HPV vaccine candidates were designed to elicit the production and activation of T cells to develop cytotoxic anti-tumor specific responses by targeting the E6 and/or E7 oncoproteins. Current therapeutic vaccine strategies include: live vector-based (viral vectors/bacterial vectors), nucleic acid-based (DNA/RNA), cell-based (dendritic cells/tumor cells), protein-based, and peptide-based vaccines. Among the various immunotherapeutic delivery systems, peptide-based vaccines are simple, stable, well tolerated and can be tailored to produce the desired immunogenic effects (Figure 1) [20-22]. Since T cells can recognize tumor-associated antigens in the form of short peptides bound to major histocompatibility complex (MHC) molecules, vaccination with peptides derived from HPV antigens provides a feasible strategy for immunotherapy against HPV infections [23, 24]. Identification of tumor-reactive T lymphocyte peptide epitopes is necessary before a vaccine can be developed. Peptide-based vaccines have the potential advantages of combining multiple epitopes to enhance peptide-MHC binding and improve specific T cell-mediated immunity against HPV-infected cells [25]. However, selection of the most appropriate T cell epitopes able to elicit responses by antitumor cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (HTLs) remains a challenge. Another limitation is that peptide-based vaccines have low immunogenicity. Many developments in the use of peptide-based vaccines have focused on enhancing vaccine potency by using adjuvants (immune stimulating agents) to circumvent this problem [26]. This review summarizes the prospects of peptide-based vaccines for the treatment of established HPV infections. We address the obstacles that scientists currently face in developing peptide-based vaccines and explore feasible strategies for improving the potency of inducing immune responses leading to the treatment of HPV infections.

**The biology of HPV infection in the cervix**

In order to develop an optimal therapeutic vaccine against HPV, it is necessary to fully understand the progression from HPV infection to cervical cancer. HPV belongs to a genus in the Papovavirus family and is a non-enveloped, double-stranded, closed circular DNA virus. The HPV genome is approximately 8,000 base pairs, encoding six early proteins (E1, E2, E4, E5, E6, and E7) and two late proteins (L1 and L2) [27]. The early genes encode non-structural proteins, regulating viral gene replication and transformation, whereas two late
genes (L1 and L2) encode the viral capsid proteins. E1, E2, and E4 proteins contribute to viral gene replication, transcription, and genome amplification, respectively. The E5, E6, and E7 proteins interact with growth factor receptors, binding to and inactivating the tumor suppressor gene products p53 and retinoblastoma (pRb), respectively [28, 29]. Interactions between HPV proteins and the host cell result in dysregulation of cell cycle control, eventually leading to the development of cervical cancer.

The infection of the cervical epithelium with HPV is closely associated with the maturation of keratinocytes (Figure 2). After infection with HPV in the basal layer of the epithelium, the viral early proteins are expressed, and interact with cellular proteins to regulate viral replication. Next, the infected basal cells move up from the basement membrane to the upper epithelial layers as epithelial cell differentiation occurs, expressing E4 protein for viral amplification. Finally, a subset of E4-positive cells express the late proteins L1 (major) and L2 (minor), and L1 and L2 assemble to form the new infectious virions. Therefore, mature virions are released only by the superficial epithelial keratinocytes [27].

Integration of the viral episome into the host DNA occurs in cells infected with high risk HPV. This often results in the deletion or inactivation of some early (E2, E4 and E5) and late (L1 and L2) genes, while the E6 and E7 genes are consistently expressed within infected cells [13, 14]. The viral E2 gene is a negative regulator of E6 and E7 expression. Subsequently, interruption of E2 gene leads to over expression of the E6 and E7 genes [30]. In addition, the E6 and E7 genes are able to bind and complex with the tumor suppressor gene products, p53 and retinoblastoma (pRb) protein, respectively. p53 and pRb have tumor-suppressive and cell cycle growth inhibitory functions [31, 32]. Thus, over-expression of E6 and E7 oncoproteins results in the dysregulation of the cell cycle, causing cellular transformation which leads to the development of cervical intraepithelial neoplasia (CIN) that may eventually progress to cervical cancer [33].

**Humoral immune responses and cell-mediated immune responses to HPV infections**

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It is estimated that over 98% of HPV infections resolve spontaneously. Nevertheless, the natural immune response to HPV after infection is weak, and the clearance of infected cells is much slower, when compared with that of most other viral infections [34, 35]. Animal studies indicated that, when expressed, the major capsid protein (L1) of HPV elicits virus-neutralizing antibodies that can protect the host from HPV infection. Humoral antibody responses to the capsid proteins were weak, and measurable immune responses were only observed in about 50% of subjects with a persistent HPV infection [36]. The L1 (major) capsid protein has been used to produce virus like particles (VLPs), currently employed in the marketed prophylactic anti-HPV vaccines. According to serological studies based on VLPs, in about 50% of patients the HPV infection causes a humoral response (IgG) against conformational epitopes of the L1 (Major) capsid protein [37, 38], although the duration of measurable humoral immunity to HPV capsid proteins following viral clearance is unknown. Unfortunately, immunization with capsid proteins failed to induce powerful therapeutic effects for established HPV infections that had evaded antibody-mediated neutralization [39]. It is likely that this is because the capsid proteins are only expressed in the superficial epithelial keratinocytes, but not in basal keratinocytes. Persistent HPV infection is very prevalent and contributes to increased morbidity and mortality. An effective vaccination strategy is important in order to treat and eradicate cells already infected with HPV.

Vaccines that elicit cell-mediated immune responses to nonstructural viral proteins are, in principle, more likely than antibody-mediated neutralization to cause the regression of established lesions or even cancers. HPV infection frequently induces discrete humoral and cell-mediated immune responses [40]. Evidence suggests that cellular immunity, particularly a cellular infiltrate of antigen-specific T cells, is associated with regression of established HPV infection [41, 42]. Several observations indicate that cell-mediated immune responses are important for the control and eradication of established HPV infection [43]:

1. Humoral immunodeficiency does not predispose HPV-positive patients to the growth of HPV-associated, pre-cancerous lesions.
2. The prevalence of HPV-associated disease is increased in patients with reduced CD4+ T cell function, such as transplant recipients [44] and human immunodeficiency virus (HIV)-infected patients [45, 46].

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(3) One study indicated that spontaneous regression of CIN is accompanied by a delayed-hypersensitivity response to the viral E7 protein mediated by CD4+ T cells infiltrating the infected epithelium [47].

(4) Cervical tumors contain functionally altered dendritic cells (DCs) leading to inhibition of CD8-mediated antigen-specific T cell responses [48].

(5) Immunostimulatory functions mediated by cytokines are disabled in HPV-induced tumors [49].

These observations support the hypothesis that, although humoral immunity induces antibody-mediated neutralization to protect the host against initial infection, cell-mediated immunity is necessary for the eradication of HPV infection and inhibition of tumor progression. Therefore, to be effective, a therapeutic HPV vaccine must be able to stimulate enhanced HPV-specific cell-mediated immune responses.

The host antigen presenting cells (APCs) are an appropriate target for a therapeutic HPV vaccine (Figure 1). Many studies report that professional APCs (including dendritic cells, macrophages, and B cells) were critical components in mediating immunotherapy. Dendritic cells (DCs) are potent professional APCs that initiate epithelial immune responses by priming helper T cells and killer T cells *in vivo* [50-52]. Langerhans cells (LCs), specialized DCs of the epidermis, capture antigens and present large numbers of immunogenic MHC-peptide complexes on their surface [53, 54]. These, LCs migrate to the lymph nodes where they present antigen collected in the epithelium directly to the antigen-specific T cells [55]. DCs highly express MHC-I and MHC-II molecules and co-stimulatory molecules that are involved in antigen-specific T cell activation. DCs up-regulate the expression of MHC-I and MHC-II molecules and adhesion molecules and become more potent stimulators of T cell-mediated immunity through maturation-inducing stimuli such as inflammatory cytokines [39]. Effective therapeutic vaccines must have a two-fold effect: targeting tumor antigens to professional APCs, and enhancing MHC-I and/or MHC-II presentation of the tumor antigen to activate antigen-specific T cells.

Persistent HPV infection is a consequence of the natural anti-cancer immunity failing to eliminate the infected cells [56]. The HPV early proteins play a pivotal role in evasion of the host immune system through a variety of mechanisms:

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(1) The HPV-16 E7 oncoprotein has high homology with several human proteins [57], which may facilitate recognition as autoantigen.

(2) The E6 oncoprotein inhibits the interactions between epithelial cells and DCs [58], possibly accounting for the depletion of DCs observed in the HPV-infected cervical epithelium [59]. An E6 oncoprotein-mediated reduction in expressed interleukin-8 (IL-18) CD8+ was also observed [60].

(3) The E6 and E7 oncoproteins down-regulate the production and responsiveness of infected cells to type 1-interferons (IFNs) [61, 62]. Both E6 and E7 oncoproteins inhibit IL-18-induced IFN-γ in natural killer (NK) cells [63]. Additionally the E7 oncoprotein arrests the antiviral signaling mediated by interferon-alpha (IFN-α) [64, 65] and has also been observed to inhibit the IRF-1-mediated activation of the IFN-β promoter [66].

(4) Extracellular E7 oncoprotein potentially has the ability to inhibit T cell responses [67].

(5) The E5 oncoprotein binds the 16-kDa subunit of vacuolar proton-ATPase and disturbs its activity, inhibiting pH-dependent processing of antigenic peptides, which may interfere with proper antigen presentation [68-70].

Cervical cancer has been shown in the past to inactivate the type 1 interferons (IFNs), cytokines such as IL-18, and antigen processing and presentation. A recent study has revealed that cervical extracts, in particular the protease component, can suppress lympho-proliferative responses [71], whereas expression of FasL on cervical tumors can cause apoptosis of the infiltrating lymphocytes [72]. These results also indicate that the production of regulatory T cells may be an important suppressive mechanism in cervical cancer [73, 74], because regulatory T cells suppress the induction of type 1-helper T (Th1) cells [75], and IFN-γ-producing Th cells are required to mobilize CD8+ T cells to the site of HPV infection [76]. Therefore, a successful immunotherapeutic approach to cervical cancer must circumvent the activity of these cell types and other local immunosuppressive mechanisms.

Therapeutic HPV vaccine development

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Therapeutic vaccines that target established HPV infections aim to generate specific cell-mediated immunity that eliminates pre-existing lesions and malignant tumors. A therapeutic vaccine should target HPV antigens that are constantly expressed in the infected cells. Thus, in contrast to the prophylactic HPV vaccines, therapeutic HPV vaccines need to contain an antigenic determinant derived from HPV early proteins, such as the E6 and E7 proteins, which are expressed throughout the life cycle of HPV [29, 77-80]. Since E6 and E7 oncoproteins are required for the induction and maintenance of the malignant phenotype of cancer cells [81], cervical cancer cells are unlikely to escape immune attack targeting these proteins via antigen loss. The E7 protein is more abundantly expressed and is more highly conserved than the E6 protein [82, 83]. The E7 protein also induces genotype-specific antibody after the commencement of invasive cervical cancer, thus the E7 proteins can generate therapeutic and protective effects [82]. Other early viral proteins, the E1, E2, E4, and E5 proteins, also have potential as therapeutic vaccine antigens for the treatment of warts. However, neither the E1 protein nor the E2 protein is constantly expressed in HPV-induced carcinoma. Evidence suggests that E5 may play a critical role in the genesis of cervical cancer but is less important in persistence and progression [84]. Therefore, the E5 protein has limited immunogenicity and has not been extensively researched as a promising vaccine antigen [85]. Similarly, the E4 protein and L1 and L2 capsid proteins are unlikely to be proper targets for therapeutic vaccine antigens because these proteins are not expressed at detectable level in infected basal epithelial cells and cervical cancer cells [86].

In order to treat patients with pre-invasive and invasive cervical cancers, therapeutic vaccines need to elicit specific cytotoxic anti-tumor responses by targeting CD8+ T cell activation via MHC-I restricted antigens. Additionally, CD4+ T cell responses, which are MHC-II restricted, are equally important in both effective anti-viral and antitumor CTL responses, and lead to destruction of the virus or tumor by CD8+ CTLs [87, 88]. The function of CD4+ T cells in the priming phase of a tumor specific CTL response is thought to occur at the level of activating DCs to allow these cells to effectively activate naive CD8+ T cells [89-91]. Consequently, several requirements need to be met for the establishment of an effective therapeutic vaccine against cervical cancers:

1. The target antigen must be recognized by the T cells rather than by antibodies.
2. The vaccine should target appropriate APC subsets.
(3) The tumor antigen should induce large number of effector T cells, including both CD4+ and CD8+ T cells.

(4) Ensure efficient T cell trafficking to the tumor site.

(5) Circumvent the activity of local immunosuppressive mechanisms at the tumor site.

(6) Induce strong, long-lasting inflammation at the tumor site.

Several HPV vaccine strategies have been devised to achieve these requirements with different degrees of success. Peptide-based vaccines are one of the most promising approaches to develop a safe and efficient therapeutic vaccine to treat HPV-related cancers.

**Peptide-based HPV vaccine development**

Synthetic peptide-based vaccines are potentially excellent candidates for rational vaccine development because they are naturally non-infectious, completely defined, relatively easy to produce, and are generally considered to be safe. Peptide-based vaccines are well characterized, stable in freeze-dried form, and are able to combine multiple epitopes [20, 22, 25]. However, their low immunogenicity must first be overcome. Another limitation of peptide-based vaccines is the need to match the patient’s human leukocyte antigen (HLA). HLA polymorphisms in patients make it difficult to develop a peptide-based vaccine that is applicable to the whole population. In the case of peptide-based vaccines against HPV, several specific CTL epitopes from HPV-16 E6 and E7 have been characterized for the HLA-A2 molecule, the most common MHC-I molecule in humans [92-94]. Immunization with a peptide carrying MHC-I restricted epitopes from E6 and/or E7 have been shown to elicit cell-mediated immune responses in both animal and human models [92, 94]. In addition to finding an epitope that interacts with the host HLA, the design of a peptide-based anti-HPV vaccine relies on the accurate identification of an appropriate protective epitope.

**1. Epitope selection**

Epitope identification and selection of most relevant epitopes are the initial challenges of peptide-based vaccine development. In one of the first anti-HPV peptide studies, Feltkamp et al. identified the sequence E749-57 (RAHYNIVTF) as an MHC-I binding peptide that can elicit CTL responses in HPV 16-induced tumor tissue [95, 96]. Tindle et al. identified E748-54 (DRAHYN) as a Th-cell-stimulating epitope that helped to elicit antibody responses to

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HPV-16 E7 in a mouse model [97]. Recently, multiple studies have indicated that the use of longer versions of CTL peptide epitopes, covering the Th-cell epitope, ensures the induction of sustained CD8+ T cell reactivity in vivo, when compared with using the minimal CTL peptide epitope (E749-57) [98, 99]. Extension of the CTL peptides to longer variants may offer an excellent alternative when external specific CD4+ helper T cell epitopes are not readily available [99]. A recent attractive approach for vaccination has been the use of overlapping long peptides as antigens. The use of overlapping long peptide vaccines that cover the whole HPV E6/E7 sequence, representing the complete antigen in several fragments, circumvented the need to target a patient’s HLA type. This approach led to more efficient peptide presentation, minimizing peptide-induced tolerance via antigen presentation by non-professional APCs. This approach has been tested in preclinical mouse [100] and rabbit [98] models, and in clinical trials [101-103].

2. Vaccine delivery

In general, peptide-based vaccines have weak immunogenicity, but the use of adjuvants (immune stimulating agents) can boost the immunogenicity of these vaccines [22]. A number of adjuvants induce strong immune responses and are widely used in animal models (e.g. incomplete Freund’s adjuvant, IFA) [95, 100, 104]. However, most experimental adjuvants are unsuitable for human use because of serious adverse side effects. Some oil-emulsion adjuvants have been approved and commonly employed for use in human trials (e.g. Montanide ISA 51, a human grade IFA-like adjuvant) [101-103, 105-107]. Another strategy to enhance the potency of peptide-based vaccines is the conjugation of immunostimulatory lipids to a peptide antigen. Lipidic carriers have been used extensively for vaccine delivery and can act as self-adjuvanting moieties [108-111]. The lipid core peptide (LCP), a vaccine delivery system incorporating lipoamino acids, was used to produce vaccine candidates against HPV-16 [104]. Moyle et al. demonstrated that an LCP system that incorporated the long sequence E744-62 (known as 8Q) with CTL, T helper cell, and B cell epitopes was self-adjuvanting and reduced tumor size. This epitope was unable to prevent TC-1 tumors when delivered alone [104]. The self-adjuvanting properties of LCP are predominantly derived...
from its propensity to target vaccines to DCs and induce DC maturation [22, 112]. In another mouse study, a lipopeptide vaccine incorporated the E643-57 and E744-62 epitopes conjugated to a dipalmitoyl-lysine-glycine-glycine (Pam2KGG) moiety. It was shown that although both epitopes were individually able to elicit specific CTL responses in mice, the lipid-tailed (Pam2KGG) diepitopic construct was more efficient than the monomeric forms [113].

Liposomes have been used extensively to deliver low molecular weight drugs, plasmid DNA, oligonucleotides, proteins, and peptides as well as for the delivery of peptide-based antigens. Huang and coworkers developed a liposome-based, nanoparticle delivery system, called Liposome-Protamin-DNA (LPD) [114]. LPD is a self-assembled mixture of cationic liposomes, polycations, and plasmid DNA. In their early studies, the LPD/E7 formulation (which contains bacterial DNA) showed the potential to eradicate established E7-expressing TC-1 tumors. Afterwards, they employed cationic 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) as a potent cancer vaccine adjuvant to form an DOTAP/E7 complex, which contained the DOTAP cationic lipid and the E749-57 peptide antigen. The DOTAP/E7 formulation has the ability to elicit the activation of DCs without plasmid DNA, generate antigen-specific CD8+ T lymphocyte responses, and eradicate established E7-expressing TC-1 tumors after a single immunization. Huang’s group has improved vaccine formulation by incorporating an E7-lipopeptide, instead of the native E7 peptide, into the DOTAP liposome [REF]. The lipopeptide consists of an N-terminal α- or ε-palmitoyl lysine connected to the E7 peptide via a dipeptide Ser-Ser linker. The presence of the dipeptide linker sequence between the E7 peptide and the attached fatty acid was necessary to stimulate a full immune response. The DOTAP/E7-lipopeptide formulation was more than twice as potent as the DOTAP/native E7 formulation [114]. Another liposome strategy employed an adjuvant in which gangliosides are incorporated into the outer membrane protein complex of Neisseria meningitides to form very small size proteoliposomes (VSSP) for DC activation and Th 1 differentiation. This was the first study to show that VSSP with a minimal CTL (E749-57) epitope could be used as an anti-cancer immunotherapy by inducing an E7-specific CD8+ T cell response [115].

Other popular adjuvants used for peptide-based vaccines include two strong DC-activating agents: an oligodeoxynucleotide (ODN)-CpG [85, 100, 116-118] and a monophosphoryl lipid...
A (MPL) [100]. A recombinant cytokine, e.g. granulocyte-macrophage colony-stimulating factor (GM-CSF), can be used as an adjuvant for augmenting immunogenicity in peptide-based vaccines [100, 119]. In one of the latest studies, a self-assembled nanoparticle HPV vaccine was designed to combine the cell-penetrating peptide, HIV-1 Tat 49-57, that was fused with the minimal CTL (E7_{49-57}) epitope and GM-CSF[119]. This vaccine generated potent, long term, anti-tumor immune responses through the production of long lasting memory CD8\(^+\) cells. Another pre-clinical study employed an E7_{43-77} long peptide, containing both CTL and Th epitopes, and several DC-activating adjuvants, including GM-CSF, ODN-CpG and MPL, for the optimal priming of tumor-specific CTLs. Vaccination with the long peptide led to the induction of CD8\(^+\) CTL and CD4\(^+\) Th cells. CD4\(^+\) Th cells contribute to the level of the induced E7-specific CD8\(^+\) CTL cells responses by delivering essential activation signals to DCs. It was suggested that the long peptide was preferentially endocytosed, processed and presented by professional APCs, and this could circumvent the potential hazard of peptide-induced tolerance. The potency of the vaccine was further augmented by the use of DC-activating adjuvants, notably ODN-CpG. Synthetic ODN-CpG mimics bacterial DNA and binds toll-like receptor (TLR)-9 providing a “danger signal” to activate the immune response [120]. Therefore, vaccination with a long peptide containing CD8\(^+\) CTL and CD4\(^+\) Th cell epitopes, and DC-activating agents could augment the immunity generated against peptide-based HPV vaccines [100]. A similar long peptide and adjuvant combination was also trialed in the cottontail rabbit papillomavirus (CRPV) preclinical model of persistent HPV infection. Overlapping long E6 and E7 peptides, which contained both CD4\(^+\) T helper and CD8\(^+\) CTL epitopes, administered with the mixture of Montanide ISA 51 and ODN-CpG were tested for therapeutic efficacy against CRPV-infected lesions. After vaccination, this overlapping long vaccine was able to significantly control wart growth in rabbits [98]. Another animal study, which used a long peptide (E7_{44-62}) and the DC-activating adjuvant ODN-CpG, has identified that vaccination with a longer peptide containing CTL and Th cell epitopes, and ODN-CpG represents a promising strategy against MHC-I deficient tumors. This study also investigated the efficacy of cellular vaccines based on \textit{ex vivo} cultured DCs pulsed with either minimal CTL (E7_{49-57}) epitope or longer CTL (E7_{44-62}) epitopes, and then matured with ODN-CpG. The use of \textit{ex vivo} cultured DCs is particularly suitable for the delivery of peptide-based vaccines. Peptide antigens directly loaded onto the \textit{ex vivo} activated autologous DCs can bypass the processing requirement and allow the accurate delivery of
peptide antigens to trigger immune responses. It was revealed that longer peptides presented by \textit{ex vivo} cultured DCs resulted in stronger inhibition of tumor growth than the shorter epitopes [116].

In recent years, several novel vaccine delivery platforms were developed for augmenting the immunogenicity of peptide-based vaccines. Daftarian and coworkers encapsulated antigens and adjuvants in multilamellar liposomes in a water-in-oil emulsion, VacciMax\textsuperscript{®} (VM) [117]. VM is a liposome-based antigen delivery platform containing the Pan HLA-DR epitope (PADRE) and ODN-CpG as adjuvants and Montanide ISA 51 as the oil carrier of the water-in-oil emulsion. PADRE, a universal T helper epitope, was found to bind to different MHC-II molecules with high-affinity and was used in conjunction with other types of vaccines to increase vaccine potency in preclinical studies [121, 122]. In a preclinical study using the C3 tumor model, a single administration of a minimal CTL (E\textsubscript{749-57}) epitope via VM liposome-based antigen delivery induced a long-lasting CTL response, complete protection against tumor challenge, and rapid tumor eradication. All mice that received this vaccine remained tumor-free when re-challenged with C3 cells [117]. To extend these results to a more clinically appropriate HPV cancer model, TC-1/A2 tumor cell lines in aged HLA-A2 transgenic mice (48-58 weeks old) were employed for further study. A VM formulation containing peptide antigens derived from E7 and E6 as either a physical mixture or chemically conjugated generated a strong CTL response. The VM-formulated therapeutic vaccine induced potent immune responses that could eradicate large transplanted tumors (> 700 mm\textsuperscript{3}) in aged mice less than three weeks post-immunization with a single vaccination. Therefore, VM is a promising vaccine delivery platform for the therapeutic treatment of cervical cancer [118].

Another animal study, using a similar strategy, combined the PADRE peptide (to enhance CD4\textsuperscript{+} Th cells responses) and a TLR ligand (to enhance DC activation) to boost the immunogenicity of peptide-based vaccines [123]. Wu and co-workers designed a therapeutic vaccine combining PADRE, polyriboinosinic:polyriboctidylic acid (poly(I:C)), a toll-like receptor 3 ligand, and a minimal CTL (E\textsubscript{749-57}) epitope. In comparison to peptide with PADRE or Poly(I:C) alone, this combination elicited a high CD8\textsuperscript{+} T cell response and improved the therapeutic anti-tumor effects against TC-1 tumor. Intratumoral administration
with E7 peptide in combination with PADRE and poly(I:C) generated better CD8+ T cell immune responses when compared with subcutaneous vaccination [123]. Combining a long CTL (E43-62) epitope with poly(I:C) and the vascular disrupting agent 5,6-dimethylxanthene-4-acetic acid (DMXAA), has also been shown to enhance the E7 specific CD8+ T cell immune responses. Interestingly, the timing/treatment regimen of DMXAA administration played an important role in vaccine efficacy [124]. The transmission of HPV-16 is mainly via the genital mucosal route, thus mucosal immune responses are of interest when developing vaccination strategies against HPV. The therapeutic vaccine that co-delivered peptides from HPV-16 E744-62 and E643-57 proteins, along with the non-toxic mucosal adjuvant CT-2+ generated strong systemic and mucosal cellular immunity, along with anti-tumor efficacy [125].

3. Human trials of immunotherapy for HPV infection

Several peptide-based therapeutic HPV vaccines have been evaluated to be safe and well tolerated in early phase I/II clinical trials (Table 1) [105-107, 126]. In a phase I clinical study, 12 patients with refractory cervical cancer were vaccinated with an E7-derived lipopeptide (HPV-16 E786-93) linked to PADRE. After four vaccinations, neither adverse side effects nor clinical responses were observed in patients [126]. Phase I/II trials have also tested two HPV-16 peptides, E711-20 and E786-93, and a PADRE emulsion in the Montanide ISA 51 adjuvant in patients with recurrent or refractory cervical cancer [105, 106]. This therapeutic vaccine showed no significant adverse effects in both trials. In the phase I study, 2 out of 19 patients showed tumor-regression after chemotherapy following vaccination [105]. In the phase II trial, 2 out of 15 patients had diseased states that were unchanged more than 1 year after vaccination [106]. Based on these studies, it was suggested that vaccination of patients with less advanced cervical disease might be more effective [105, 106]. Therefore, a vaccine candidate in patients with earlier stages of HPV-induced disease was tested in a phase I trial [107]. A total of 18 patients with HPV-16-positive high-grade CIN or vulvar intraepithelial neoplasia (VIN) were vaccinated with the HPV-16 E712-20 peptide in Montanide ISA 51 and the E786-93 lipopeptide linked to PADRE. None of the patients demonstrated a delayed type hypersensitivity (DTH) response. In this case, 3 of 18 patients were free of dysplasia after vaccination and 6 patients had partial remissions. HPV E7-specific T cell immunity was detected in peripheral blood mononuclear cells from 10 out of 16 patients and an increased
S100+ dendritic cell infiltrate was observed in 6 out of 6 patients tested. Overall, these peptide-based vaccines were more effective in women who had early stage cervical cancer than in women whose immune systems were compromised by progressive disease [107].

A strategy that enhances immune responses to peptide-based vaccines by using a long overlapping peptides vaccine has been tested in early phase clinical trials. One such vaccine consisted of HPV E6 and E7 peptides (25-35 amino acids long with overlapped 10-14 amino acids) and was formulated in Montanide ISA 51 adjuvant. This vaccine was evaluated in 43 patients with end-stage cervical cancer. The vaccine was well tolerated, and stimulated the production of IFN-γ-associated T cell responses [101]. The same vaccine was used in a phase II trial with six patients with resected HPV-16 positive cervical cancer. This work also showed that this vaccine increased the activity and production of HPV-16-specific CD4+ and CD8+ T cells in all six patients [102]. Furthermore, this vaccine was tested in a phase II trial in 20 women with HPV-16-positive grade 3 VIN. The overlapping peptides vaccine elicited T cell responses and a complete clinical response in 9 out of 19 patients after the first 12 months. These responses were maintained at 24 months follow-up [103]. Overall, the overlapping long peptides vaccine offers a promising strategy for the treatment of women who are in earlier stages of HPV-induced disease.

**Conclusion**

Although commercial preventive or prophylactic HPV vaccines are now available and have the potential to prevent the onset of cervical cancer and other HPV-associated malignancies, it is predicted that it will take decades to significantly reduce the prevalence of cervical cancer. Additionally, the full effect of the new prophylactic HPV vaccines on cervical cancer incidence can only be evaluated decades after implementation. Thus, development in the meantime of a therapeutic HPV vaccine that is safe, economical, and effective is a priority.

The most promising therapeutic vaccine candidates are based on the generation of cell-mediated immune responses to eliminate HPV infection. Based on the present understanding of the molecular progression of cervical cancer, the E6 and E7 oncoproteins are the currently

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preferred targets for antigen-specific cellular immunity, since they are constitutively expressed in virtually all cervical cancer cells and are essential for the induction and maintenance of HPV-associated disease. Synthetic peptide-based vaccines have been extensively studied for the treatment of HPV infection owing to their safety, stability, and ease of production. However, many peptide-based vaccines had struggled with epitope identification and poor immunogenicity. To address these problems, short and long peptides, containing CTL and HTL epitopes or with overlapping coverage of the complete E6 and/or E7 sequence, have been used to improve the efficacy of peptide-based vaccines. Moreover, different categories of adjuvants and novel delivery systems were developed and employed for peptide-based vaccine design to enhance immunogenicity. A number of strategies for peptide anti-HPV vaccine design have been evaluated in early-phase clinical trials, and encouraging results in preclinical animal studies illustrate the promise of such approaches. Perhaps the most promising strategies have involved the use of long peptides (including CD4+ and CD8+ epitopes) or a combination of overlapping peptides with a variety of immunogenic delivery systems. However, further developments in delivery and adjuvant systems will be required to produce a therapeutic anticancer peptide vaccine suitable for widespread use in humans.
Abbreviations

APC: Antigen presenting cell
CIN: Cervical intraepithelial neoplasia
CRPV: Cottontail rabbit papillomavirus
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
DMXAA: 5,6-Dimethylxanthenone-4-acetic acid
DOTAP: 1,2-Dioleyoyl-3-trimethylammonium propane
DTH: Delayed type hypersensitivity
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HLA: Human leukocyte antigen
HPV: Human papillomavirus
HTL: Helper T lymphocyte
IFA: Incomplete Freund’s adjuvant
IFN: Interferon
IL: Interleukin
IRF: Interferon regulatory factor
LC: Langerhans cell
LCP: Lipid core peptide
LPD: Liposome-Protamin-DNA
MHC: Major histocompatibility complex
MPL: Monophosphoryl lipid A
NK cell: Natural killer cell
ODN-CpG: Oligodeoxynucleotides (ODN)-CpG
PADRE: Pan HLA-DR epitope
Pam2-KGG: Dipalmitoyl-lysine-glysine-glysine
Poly(I:C): Polyriboinosinic:polyribocytidylic acid
pRb: Retinoblastoma
Th cell: Helper T cell
VIN: Vulvar intraepithelial neoplasia
VLP: Virus-like particle
VM: VacciMax®

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Figure (1). Relationship between antigen presenting cells and humoral and cellular immunity

(a) APCs uptake the immunogen, process it into peptide epitopes, and present it on MHC class I and/or class II molecules on the cell surface. T cell receptors of T helper cells (CD4+ T cells) interact with the Th-epitope/MHC II complex. (b) Following activation of Th cells and B cell maturation, antibody secretion occurs. (c) Naive CD8+ T cells can be stimulated through the interaction of activated Th cells with certain types of APC. (d) CTLs have the ability to recognize and kill target cells that display viral or tumor peptides. These CTLs are generated from naive CD8+ T cell through the interaction with activated APC carrying above peptide epitopes on MHC class I molecules [25].
Figure (2). Schematic representation of HPV gene expression during keratinocyte differentiation in the stratified squamous epithelium. After infection with HPV in the basal layer of the epithelium, early viral proteins (E1, E2, E4, E6, and E7) are expressed. As the keratinocytes mature and progress through the epithelium, the late proteins (L1 and L2) assemble to form the new infectious virions, and mature virions are produced only in the most superficial layers of the epithelium.
Table 1. Summary of clinical trials for peptide-based therapeutic HPC vaccine candidates.

<table>
<thead>
<tr>
<th>Vaccine composition/Adjuvant</th>
<th>Target antigen(s)</th>
<th>Phase</th>
<th>Patients</th>
<th>Immune response</th>
<th>Clinical response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7_{86-93} lipopeptide linked to PADRE</td>
<td>HPV-16 E7</td>
<td>I</td>
<td>12 patients (HLA-A2-positive) with recurrent or persistent cervical cancer (HPV-16-positive)</td>
<td>CTL response in 7 patients</td>
<td>No clinical response</td>
<td>[126]</td>
</tr>
<tr>
<td>E7_{11-20}, E7_{86-93} and PADRE emulsified in Montanide ISA 51 adjuvant</td>
<td>HPV-16 E7</td>
<td>I/II</td>
<td>19 patients (HLA-A2-positive) with recurrent or residual cervical cancer (HPV-16-positive)</td>
<td>NO CTL response</td>
<td>2 stable disease; 2 showed tumor-regression after chemotherapy following vaccination</td>
<td>[105]</td>
</tr>
<tr>
<td>E7_{12-20} in Montanide ISA 51 and E7_{86-93} lipopeptide linked to PADRE</td>
<td>HPV-16 E7</td>
<td>I</td>
<td>18 patients (HLA-A2-positive) with high grad CIN/VIN (HPV-16-positive)</td>
<td>CTL response in 10 patients; No DTH</td>
<td>3 complete responses; 6 partial responses</td>
<td>[107]</td>
</tr>
<tr>
<td>Overlapping long peptide (nine E6 and four E7 peptides of 25-35 amino acids long with an overlap of 10-14 amino acids) emulsified in Montanide ISA 51</td>
<td>HPV-16 E6/E7</td>
<td>I</td>
<td>35 end-stage cervical cancer patients</td>
<td>IFN-γ T cells induced</td>
<td>Not determined</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>6 patients with resected cervical cancer (HPV-16-positive)</td>
<td></td>
<td>Not determined</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>20 patients with grade 3 VIN (HPV-16-positive)</td>
<td></td>
<td>9 complete responses; 6 partial responses</td>
<td>[103]</td>
</tr>
</tbody>
</table>

Abbreviations: PADRE: Pan HLA-DR epitope; HPV: Human papillomavirus; HLA: Human leukocyte antigen; CTL: Cytotoxic T lymphocyte; VIN: Vulvar intraepithelial neoplasia; DTH: Delayed type hypersensitivity; IFN: Interferon.

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