REVIEW ARTICLE

Biodrugs 2010; 24 (2): 109-129
1173-8804/10/0002-0109/$49.95/0

Immunotherapy for Cervical Cancer

Research Status and Clinical Potential

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Abstract

The high-risk types of human papillomavirus (HPV) have been found to be associated with most cervical cancers and play an essential role in the pathogenesis of the disease. Despite recent advances in preventive HPV vaccine development, such preventive vaccines are unlikely to reduce the prevalence of HPV infections within the next few years, due to their cost and limited availability in developing countries. Furthermore, preventive HPV vaccines may not be capable of treating established HPV infections and HPV-associated lesions, which account for high morbidity and mortality worldwide. Thus, it is important to develop therapeutic HPV vaccines for the control of existing HPV infection and associated malignancies. Therapeutic vaccines are quite different from preventive vaccines in that they require the generation of cell-mediated immunity, particularly T cell-mediated immunity, instead of the generation of neutralizing antibodies. The HPV-encoded early proteins, the E6 and E7 oncoproteins, form ideal targets for therapeutic HPV vaccines, since they are consistently expressed in HPV-associated cervical cancer and its precursor lesions and thus

play crucial roles in the generation and maintenance of HPV-associated disease. Our review covers the various therapeutic HPV vaccines for cervical cancer, including live vector-based, peptide or protein-based, nucleic acid-based, and cell-based vaccines targeting the HPV E6 and/or E7 antigens. Furthermore, we review the studies using therapeutic HPV vaccines in combination with other therapeutic modalities and review the latest clinical trials on therapeutic HPV vaccines.

Cervical cancer is the second most common cause of cancer in women worldwide, with approximately 510 000 new cases and 288 000 deaths reported annually.[1] The high-risk types of human papillomavirus (HPV) have been found to be associated with the majority of cervical cancers and its precursor lesions.[2] Two high-risk types, HPV-16 and -18, account for up to 75% of all cervical cancers. The identification of HPV as the etiological factor for cervical cancer provides an opportunity to control cervical cancer through vaccination against HPV. In order to develop effective therapeutic vaccines, it is essential to have a thorough understanding of the HPV biology and its role in the pathogenesis of cervical cancer.

HPV is a non-enveloped, double-stranded, circular DNA virus with unidirectional transcription. Its genome encodes six to seven early proteins (E1, E2, E4, E5, E6, E7, and E8), depending on the type of HPV, and two late (structural) proteins (L1, L2). The life cycle of HPV is closely associated with keratinocyte maturation. In order to establish an infection, the virus needs to infect the basal epithelial cells, which are capable of active replication and differentiation. As the keratinocytes undergo differentiation, the early gene products are expressed, and interact with cellular proteins to regulate viral DNA replication. In the terminally differentiated superficial cells, the late proteins are expressed and then assemble to form the structural components of the viral capsid. In some cases, the viral DNA is integrated into the host genome. The integration of viral DNA into the host genome often results in the deletion of several early (E2, E4, and E5) and late genes (L1 and L2), and is thought to be required for the transformation of epithelial cells by HPV. The two well known HPV-encoded oncoproteins, E6 and E7, bind and complex with tumor protein p53 (TP53) and retinoblastoma 1 (RB1), respectively. Since E2 is a transcriptional repressor of E6 and E7, the loss of E2 leads to upregulation of E6 and E7, thus contributing to malignant transformation.[3-5] Through interaction with TP53 and RB1, the uncontrolled expression of E6 and E7 may cause disruption of cell cycle regulation and lead to genomic instability (for review, see zur Hausen[6]).

For the prevention of HPV infection, it is necessary to elicit an antibody response that neutralizes HPV particles prior to their entry into epithelial cells. The L1 protein on the viral capsid represents an ideal target for neutralizing antibody generation, and therefore the development of preventive vaccines. Studies have shown that expression of the recombinant major capsid protein L1 in various cell types leads to the generation of virus-like particles (VLPs) that are morphologically and immunologically similar to native virions.[7-9] Vaccination with HPV L1 VLPs has been shown to induce high titers of neutralizing antibodies in animal models and in humans (for review, see Roden et al.[10]). The newly licensed HPV preventive vaccines Gardasil® and Cervarix® represent a groundbreaking achievement in HPV vaccine development. Gardasil® is a quadrivalent L1 VLP recombinant vaccine derived from HPV types 6, 11, 16, and 18, while Cervarix® is an L1 VLP vaccine derived from HPV types 16 and 18. These vaccines generally offer type-restricted protection against cervical lesions associated with the specific types of HPV included in the vaccine, but also has some partial cross-protection against other closely related types of HPV.[11-13] Since HPV-16 and -18 account for nearly 75% of all cervical cancers, Gardasil® and Cervarix® may protect up to 80% of all cervical cancers due to their cross-protection against closely related types such as HPV-31 and -45.

Despite the success of preventive HPV vaccines, its limited availability to developing countries, where there is a high prevalence of cervical cancer, may undermine the efforts made to reduce the incidence of the disease on a global scale. The high cost and the need for refrigeration of the currently available HPV vaccines may preclude the use of these preventive HPV vaccines in the developing countries, where most of the cervical cancers occur. Thus, we may not be able to see a significant difference in incidence and prevalence of cervical cancer worldwide in a short period.[14] Another important issue in the control of cervical cancer is the treatment of established HPV infections and HPV-associated diseases. There is currently a significant burden of HPV-associated lesions worldwide, and existing preventive HPV vaccines, such as Gardasil® and Cervarix®, do not generate therapeutic effects against established HPV infection.[15] Therefore, there is an urgent need to develop therapeutic HPV vaccines for the control of existing HPV infection and associated malignancies. In the following sections, we will discuss the various forms of therapeutic HPV vaccines.

1. Therapeutic Human Papillomavirus (HPV) Vaccines

Since infected basal epithelial cells and cervical cancer cells do not express an appreciable level of L1 and/or L2 antigens, it is unlikely that therapeutic HPV vaccines targeting the L1 or L2 antigens will generate therapeutic effects against established HPV infection and HPV-associated cancer. Thus, it is important to develop therapeutic HPV vaccines targeting antigens other than L1 and L2. The HPV E6 and E7 oncoproteins represent ideal targets for the development of therapeutic HPV vaccines. These early antigens are constantly expressed in HPV-associated cancers and contribute to the progression of HPV-associated malignancies.[3] Furthermore, since HPV E6 and E7 are foreign proteins, they can circumvent the issue of immune tolerance against self-antigens, as in the case of many cancer vaccines targeting endogenous self-antigen. Therefore, in order to eradicate established HPV infections and HPV-associated cervical cancers, many investigators focused on HPV E6, E7 antigens for the development of therapeutic HPV vaccines.

Various forms of therapeutic HPV vaccines targeting HPV E6/E7 antigens have been tested in preclinical models and clinical trials. These approaches include live vector-based vaccines, protein-based vaccines, peptide-based vaccines, nucleic acid-based vaccines, and whole cell-based vaccines (see figure 1). Table I summarizes the advantages and disadvantages of each approach. The following sections outline the principles of various forms of therapeutic HPV vaccine development, and the latest results from both preclinical studies and clinical trials. Table II summarizes the significant clinical trials that have been conducted using therapeutic HPV vaccines.

1.1 Live Vector-Based Vaccines

Live vector-based vaccines usually fall into two categories: (i) bacterial vectors; and (ii) viral vectors. One important advantage of using live vector-based vaccines is their high efficiency in delivering antigens or DNA-encoding antigens of interest.

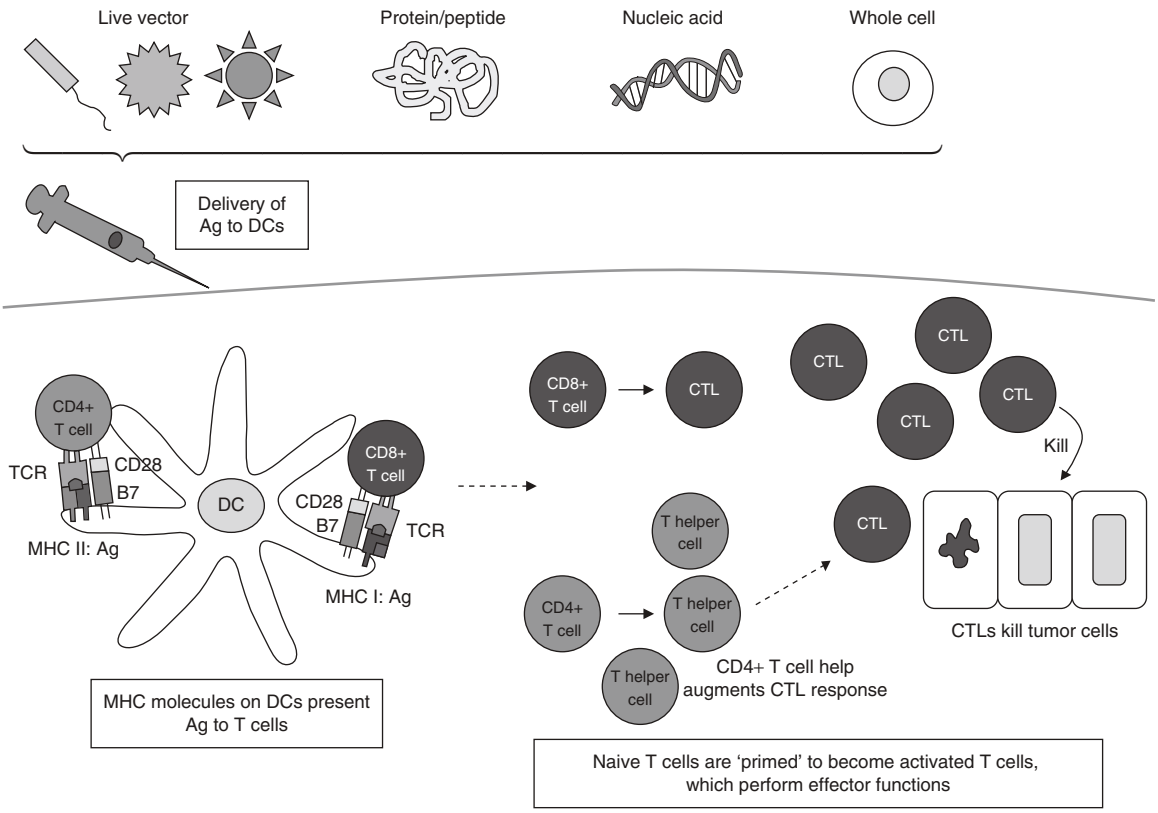

Fig. 1. Therapeutic human papillomavirus (HPV) vaccination, illustrating the immunologic effects of therapeutic vaccination with live vector-based (viral/bacterial) vaccines, protein- or peptide-based vaccines, nucleic acid-based (DNA or RNA) vaccines, or whole cell-based (tumor cell or dendritic cell [DC]) vaccines. DCs are the most potent professional antigen-presenting cells (APCs) that prime T cells *in vivo*. After uptake of antigen (Ag), DCs migrate to secondary lymphoid organs, where they present antigen to naive T cells and stimulate them to become antigen-specific T cells. Thus, many therapeutic vaccine strategies have focused on targeting antigens to professional APCs, such as DCs, and enhancing antigen processing and presentation in DCs in order to augment T cell-mediated immune responses. DCs activate the HPV antigen-specific CD4+ T cells (T helper cells) and CD8+ cytotoxic T lymphocytes (CTLs). These CTLs mediate immune clearance by apoptosis of tumor cells while T helper cells can augment CTL immune response via CD4+ T cell help. **MHC** = major histocompatibility complex; **TCR** = T-cell receptor.

Table I. Advantages and disadvantages of current therapeutic human papillomavirus vaccines

Vaccine type	Advantages	Disadvantages
Live vector-based	Many available vectors to choose for desirable effects High immunogenicity Efficient in delivering antigens to DCs Can be engineered to express immunostimulatory molecules	Risk of toxicity to patients Potential pre-existing immunity Production of neutralizing antibodies may decrease potency and inhibit repeat immunization
Peptide-based	Can combine multiple epitopes Can enhance peptides for MHC binding Stable, safe, easy to manufacture	Low immunogenicity Must determine epitopes MHC-specific; difficult to apply to a variety of patients
Protein-based	Contain all possible HLA epitopes of an antigen; no MHC specificity limitation Stable, safe, easy to manufacture Multiple known adjuvants	Low immunogenicity Usually elicit better antibody response than CTL response
DNA-based	Stable, safe Easy to produce, store and transport Capable of repeated administration Can be engineered to add targeting and/or co-stimulatory genes Variety of delivery methods Sustained expression of antigen on MHC : peptide complex	Low immunogenicity Possible chromosomal integration Lack intrinsic ability to amplify and spread to surrounding cells
RNA replicon-based	Able to replicate in transfected cells; self-limiting No possible chromosomal integration	Unstable; transfected cells may soon undergo apoptosis Difficult to store and handle Difficult to prepare large-scale Labor-intensive preparation
DC-based	High immunogenicity Multiple methods of antigen loading available	Labor-intensive; costly Challenging to establish standard criteria in vaccine preparation
Tumor cell-based	Cover various tumor antigens; tumor antigens do not have to be well defined Likely to express relevant tumor antigen	Labor-intensive preparation Safety concerns Large-scale production difficult

CTL = cytotoxic T lymphocyte; **DC** = dendritic cell; **HLA** = human leukocyte antigen; **MHC** = major histocompatibility complex.

Additionally, some live vectors can replicate and spread in the host, resulting in potent immune responses. Another advantage of live vector-based vaccines is the wide range of vectors to choose from; this makes it possible to find a desirable vector to deliver antigens. Although live vectors have many advantages, there are several drawbacks to their clinical application, including potential safety concerns for the host. In addition, the neutralizing antibodies generated against live vectors upon vaccination may limit the efficacy of repeated immunizations with the same vector. Recently, it has been demonstrated that cyclo-oxygenase-2 inhibitors such as celecoxib can prevent the generation of neutralizing antibodies to vaccinia, allowing repeated administration without losing infectivity,[59] and representing a potentially useful approach to boost the potency of viral vector-based vaccines.

1.1.1 Bacterial Vectors

Various bacterial vectors including *Listeria monocytogenes*,[60] *Lactococcus lactis*,[61,62] and *Lactobacillus plantarum*[63] have been tested in therapeutic HPV vaccines. Among the various bacterial vectors, *Listeria*-based vectors represent a potential promising vector for therapeutic HPV vaccine. *Listeria* is a Gram-positive bacterium that usually infects macrophages. Unlike most intracellular pathogens, *Listeria* is able to evade phagosomal lysis by secreting a factor called listeriolysin O (LLO) and replicating in the cytoplasm of the host cell (for review, see Schnupf and Portnoy[64]). Because *Listeria* is present in the cytoplasm and the endosomal compartments, peptides derived from *L. monocytogenes* can be presented via both major histocompatibility complex (MHC) class I and MHC class II pathways to induce potent antigen-specific T cell-mediated immune responses. Furthermore, live vector-based vaccines using *Listeria* as a bacterial vector have been proven to be able to break immune tolerance. Souders et al.[65] have shown that in HPV-16 E6/E7 transgenic mice, *Listeria*-based vaccines targeting E7 can cause regression of implanted E6/E7 expression tumors. Furthermore, it has been reported that *Listeria*-based

Table II. Selected therapeutic human papillomavirus (HPV) vaccine clinical trials

Type	Vaccine construct	Target subtype(s)	Antigen	Sponsors	Patient population	Ref.
Live vector-based	Live *Listeria monocytogenes* (Lovaxin C)	HPV-16	E7 fused to listeriolysin O protein	Advaxis Inc.	Phase I trial in 15 patients with end-stage (stage IVb) cervical cancer	16
	Recombinant vaccinia virus (TA-HPV)	HPV-16 HPV-18	E6 and E7	Xenova/Cantab (acquired by Celtic Pharma)	Phase I/II trial in 8 patients with advanced cervical cancer	17
					Phase I trial with 29 patients with stage Ib or IIa cervical cancer	18
					Phase II trial in 18 patients with high-grade VIN	19
					Phase II trial in 12 patients with high-grade VIN, VAIN, or AGIN	20
				European Organization for Research and Treatment of Cancer	Ongoing phase II trial in patients with stage Ib or IIa cervical cancer	21
	Recombinant vaccinia virus (MVA-E2)	HPV-16 HPV-18	E2	Instituto Mexicano del Seguro Social (IMSS)	Phase I/II trial in 36 patients with CIN 1–3	22
					Phase II trial in 34 patients with high-grade CIN	23
					Phase I/II trial in 50 men with flat condyloma lesions	24
	Recombinant vaccinia virus (MVA-HPV-IL-2; TG4001)	HPV-16	E6 and E7	Transgene/Roche	Plans for phase IIb trial in patients with CIN 2/3	25
Peptide-based	Lipopeptide	HPV-16	Lipidated E7 (HLA-A*0201 – restricted epitope, aa 86–93 lipopeptide)	Cytel Corporation (later Epimmune, then acquired by IDM Pharma)	Phase I trial in 10 patients with refractory cervical or vaginal cancer	26
	Peptide and montanide ISA-51 adjuvant	HPV-16	E7 epitopes (aa 11–22 and 86–93) and PADRE	Cytel Corporation (later Epimmune, then acquired by IDM Pharma), Dutch Cancer Society	Phase I/II trial in 19 patients with recurrent or residual cervical cancer	27
					Phase I/II trial in 15 patients with recurrent or residual cervical cancer	28
	Peptide and montanide ISA-51 adjuvant	HPV-16	E7 epitopes (aa 12–20 ± aa 86–93 linked to PADRE)	NCI	Phase I trial in 18 patients with CIN 2/3 or VIN 2/3	29
	Overlapping long peptide and montanide ISA-51 adjuvant	HPV-16	E6, E7	Dutch Cancer Society, ISA Pharmaceuticals	Phase I trial in 35 end-stage cervical cancer patients	30
					Phase II trial in 6 patients with resected stage 1B1 cervical cancer	31

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Table II. Contd

Type	Vaccine construct	Target subtype(s)	Antigen	Sponsors	Patient population	Ref.
	Peptide	HPV-16	E6, E7	NCI	Phase II trial in patients with metastatic or locally advanced cervical cancer	32
Protein-based	Fusion protein (TA-GW)	HPV-6	L2/E7	Xenova/Cantab (acquired by Celtic Pharma)	Phase I trial in 42 healthy male volunteers	33
					Phase IIa trial in 27 patients with genital warts	34
	Fusion protein (TA-CIN)	HPV-16	L2/E6/E7	Xenova/Cantab (acquired by Celtic Pharma)	Phase I trial in 40 healthy volunteers	35
	Fusion protein (PD-E7) and AS02B adjuvant	HPV-16	E7 linked to first 108 aa of *Haemophilus influenza* protein D	GlaxoSmithKline	Phase I/II trial in 7 patients with CIN 1 or CIN 3	36
	Fusion protein and ISCOMATRIX adjuvant	HPV-16	Recombinant E6/E7 protein	CSL Limited	Phase I trial in 31 patients with CIN 1–3	37
	Fusion protein (SGN-00101 or HSPE7)	HPV-16	E7 linked to HSP65 from *Mycobacterium bovis*	Nventa Biopharmaceuticals (recently bought by Akela Pharma)	Phase I/II trial in 15 HIV patients with high-grade AIN	38
					Phase II trial in 58 patients with CIN 3	39
					Phase II trial in 20 women with high-grade CIN	40
					Phase II trial in women with CIN 3	41
					Ongoing phase II trial in women with ASCUS/LSIL	42
	Fusion protein (SGN-00101 or HspE7) and poly ICLC adjuvant	HPV-16	E7 linked to HSP65 from *M. bovis*	Nventa Biopharmaceuticals (recently bought by Akela Pharma)	Ongoing phase I trial in women with CIN 1, 2, or 3	43
DNA-based	DNA (ZYC101)	HPV-16	E7 epitope (aa 83–95)	Eisai (formerly MGI Pharma, formerly Zycos)	Phase I trial in 12 patients with anal HSIL	44
					Phase I trial in 15 patients with CIN 2/3	45
	DNA (ZYC101a)	HPV-16 HPV-18	E6 and E7 epitopes	Eisai (formerly MGI Pharma, formerly Zycos)	Phase II trial in 127 patients with CIN 2/3	46
					Ongoing phase II/III trial in 251 patients with CIN 2/3	47
	DNA [pNGVL4a-Sig/E7(detox)/HSP70]	HPV-16	E7[a]	NCI	Phase I trial in 15 patients with CIN 2/3	48
					Ongoing clinical trial in patients with advanced HNSCC	[b]

Continued next page

Table II. Contd

Type	Vaccine construct	Target subtype(s)	Antigen	Sponsors	Patient population	Ref.
	DNA (VGX-3100)	HPV-16 HPV-18	E6 and E7	Inovio Biomedical Corporation/VGX Pharmaceuticals	Ongoing phase I trial in adult females, post-surgical or ablative treatment of CIN 2/3	49
	DNA [pNGVL4a-CRT/E7(detox)]	HPV-16	E7[a]	NCI	Plans for phase I trial in patients with CIN 2/3	c
DC-based	DC	HPV-18	E7		Case report of 1 patient with metastatic cervical cancer	50
	DC	HPV-16 HPV-18	Recombinant HPV-16 E7 or HPV-18 E7	Deutsche Forschungsgemeinschaft, BMBF	Clinical pilot study in patients with late stage cervical cancer	51
	DC	HPV-16 HPV-18	Recombinant HPV-16 E7 or HPV-18 E7	Italian Institute of Health (ISS)	Trial in 4 cervical cancer patients with recurrent disease refractory to standard treatment	52
	DC + KLH	HPV-16 HPV-18	Recombinant HPV16/18 E7	NIH	Phase I trial in 10 patients with stage Ib or IIa cervical cancer	53
	DC	HPV-16	E7	National Taiwan University Hospital	Ongoing phase I trial in patients with recurrent cervical cancer	54
Prime-boost	Prime with fusion protein (TA-CIN), boost with recombinant vaccinia virus (TA-HPV)	HPV-16 HPV-18	L2/E6/E7 (TA-CIN) + E6 and E7 (TA-HPV)	Xenova/Cantab (acquired by Celtic Pharma)	Phase II trial in 29 patients with high-grade AGIN Phase II trial in 29 patients with AGIN 3	55 56
	Prime with recombinant vaccinia virus (TA-HPV), boost with fusion protein (TA-CIN)	HPV-16 HPV-18	E6 and E7 (TA-HPV) + L2/E6/E7 (TA-CIN)	Xenova/Cantab (acquired by Celtic Pharma)	Phase I/II trial in 10 patients with high-grade AGIN	57
	Prime with DNA [pNGVL4a-Sig/E7 (detox)/HSP70] boost with recombinant vaccinia virus (TA-HPV) ± imiquimod	HPV-16 HPV-18	E7 contained in the pNGVL4a-Sig/E7 (detox)/HSP70 plasmid + E6 and E7 (TA-HPV)	NCI	Phase I trial in patients with HPV infection and CIN3	58

a E7(detox); E7 gene was mutated to abolish RB1 binding site.

b M. Gillison and T.-C. Wu, personal communication.

c C. Trimble, W. Huh, and T.-C. Wu, personal communication.

aa = amino acid; **AGIN** = anogenital intraepithelial neoplasia; **AIN** = anal intraepithelial neoplasia; **ASCUS** = atypical squamous cells of undetermined significance; **CIN** = cervical intraepithelial neoplasia; **DC** = dendritic cell; **HLA** = human leukocyte antigen; **HNSCC** = head and neck squamous cell carcinoma; **HSIL** = high-grade squamous intraepithelial lesions; **HSP** = heat shock protein; **IL** = interleukin; **KLH** = keyhole limpet hemocyanin; **LSIL** = low-grade squamous intraepithelial lesions; **NCI** = National Cancer Institute; **NIH** = National Institutes of Health; **PADRE** = Pan-DR binding T helper epitope; **RB1** = retinoblastoma 1; **Ref.** = reference; **VAIN** = vaginal intraepithelial neoplasia; **VIN** = vulvar intraepithelial neoplasia.

vaccines against E7 antigens can also limit growth of spontaneously arising HPV-16 E6/E7 expressing thyroid tumors in E6/E7 transgenic mice.[60] Many strategies have been employed to enhance *Listeria*-based vaccine potency by fusing HPV antigen with a *Listeria* protein, such as LLO[66] or ActA.[67] Recently, Maciag et al.[16] reported the first clinical use of a *Listeria*-based therapeutic HPV vaccine, using HPV-16 E7 antigen fused to a fragment of LLO. The vaccine was found to be well tolerated in end-stage cervical cancer patients who had failed prior chemotherapy, radiotherapy, and/or surgery.

L. lactis[68] has also been used for therapeutic HPV vaccine development. This non-pathogenic, non-invasive, and non-colonizing dairy micro-organism allows for controlled and targeted administration of vaccine antigens to the mucosal immune system, which stimulates systemic immune responses and induces cytotoxic T lymphocytes (CTLs) to clear infection. For example, intranasal vaccination with recombinant *L. lactis* expressing HPV-16 E7 antigen (LL-E7) and a secreted form of interleukin-12 (LL-IL-12) induced an E7-specific response in mice and also demonstrated therapeutic antitumor effects against HPV-16 E7-expressing tumors.[68] Furthermore, intranasal administration of LL-E7 was compared with *L. plantarum* expressing HPV-16 E7 (LP-E7) for their ability to generate E7-specific T cell-mediated immune responses and antitumor effects against E7-expressing tumors.[69] A greater efficacy of E7-specific immune response was observed for LP-E7 compared with LL-E7, suggesting that *L. plantarum* fits as a better vector for mucosal immunotherapy against HPV-related tumors. Another vector, *L. casei* expressing HPV-16 E7 antigen on its surface, has also been shown to greatly enhance E7-specific cell-mediated immune responses and antitumor effects in vaccinated mice.[70]

1.1.2 Viral Vectors

Recombinant viruses pose as attractive vaccine vectors for therapeutic HPV vaccination. Their high infection efficiency and excellent expression of antigens encoded by the virus in the infected cells make them an appealing choice for the delivery of HPV antigens (for review, see Hung et al.[71]). Many live viral vectors have been used for therapeutic HPV vaccine development, including adenoviruses,[72-74] adeno-associated viruses,[75] fowlpox viruses,[76] vaccinia viruses,[17-21,77,78] vesicular stomatitis viruses,[79] and alphaviruses (such as the Semliki Forest virus,[80-83] Venezuelan equine encephalitis virus,[84,85] and Sindbis virus[86]). In the following sections, we focus on adenovirus, vaccinia, and alphavirus for further discussion of their applications in both preclinical models and clinical trials.

Adenoviruses have been used for therapeutic HPV vaccines in preclinical studies. Recent studies have shown that a replication-deficient adenovirus-encoding fusion protein comprised of calreticulin (CRT) fused to E7 antigen (CRT/E7) protects mice against E7-expressing tumor challenge and exerts therapeutic effects against established tumors.[72] Adenovirus vaccine encoding chimeric hepatitis B virus surface antigen (HBsAg) fused to HPV-16 E7 protein is another alternative to induce good T-cell responses. The HBsAg/E7 fusion protein assembles efficiently into VLPs, and evokes E7 antigen-specific cellular immune responses.[73]

Vaccinia virus is a promising candidate for virus-based vaccines due to its high efficiency of infection and large complete genome. Several strategies have been used in vaccinia virus-based vaccine to facilitate the antigen processing in dendritic cells (DCs), such as fusing E7 with CRT[77] or listeriolysin O,[78] and they have been shown to elicit E7-specific immune responses in mice. In phase I/II clinical trials, a recombinant vaccinia virus expressing HPV-16/18 E6/E7 fusion protein (TA-HPV) has been tested. TA-HPV has been shown to induce HPV antigen-specific T cell-mediated immune response and some therapeutic effects in patients with late-stage cervical cancer,[18] stage Ib or IIa cervical cancer,[17] vulvar intraepithelial neoplasia (VIN),[19] and vaginal intraepithelial neoplasia (VAIN).[20] Furthermore, there is an ongoing phase II trial in patients with stage Ib or IIa cervical cancer to study the safety and immunological effects of vaccination with TA-HPV following surgery.[21] Other vaccinia virus vector-based therapeutic HPV vaccine candidates being tested in clinical trials are MVA-E2[22-24] and MVA-HPV-IL2.[25]

Alphaviruses have also been employed for therapeutic HPV vaccines. Semliki Forest virus, an alphavirus, has been shown to induce potent antigen-specific immune responses and break immune tolerance in immune-tolerant E6/E7-transgenic mice.[80] Recently, it has been reported that alphavirus vector-induced HPV-specific immune response is augmented by co-expression of IL-12.[82] Thus, viral vectors can be further modified to enhance their potency.

1.2 Peptide-/Protein-Based Vaccines

1.2.1 Peptide-Based Vaccines

Vaccination with peptides derived from HPV antigenic proteins involves the uptake of the peptide antigen by DCs and presentation of the peptide antigen in association with MHC molecules. Peptide vaccines are generally stable, easy to produce compared with protein vaccines, and safe compared with live vector-based vaccines. However, in order to develop peptide-based therapeutic HPV vaccines, it is generally necessary to identify the immunogenic epitope of HPV antigens. The

polymorphic nature of MHC molecules in the genetically outbred population makes it difficult to develop a 'one size fits all' peptide-based vaccine. The potential solution for this issue is the employment of overlapping long peptide vaccines covering HPV E6/E7 antigens. Overlapping long peptide vaccines against HPV E6 and/or E7 antigens have been tested in preclinical models, including mice[87] and rabbits,[88] and proven to be effective in generating antigen-specific T-cell responses.

In general, peptide vaccines have poor immunogenicity, but the use of adjuvants can circumvent this problem. Most studies on peptide-based vaccines have focused on enhancing vaccine potency by using adjuvants such as granulocyte-macrophage colony-stimulating factor (GM-CSF),[87] 4-1BB ligand,[89] mutant cholera toxin,[90] and CpG oligodeoxynucleotides (CpG ODN)[91,92] to enhance vaccine potency (for review, see Roden et al.[10]).

In early phase I/II clinical trials, several peptide-based HPV vaccines were found to be well tolerated.[26-29] Recently, Kenter et al.[30] conducted a phase I trial involving an overlapping HPV-16 E6 and E7 long peptide vaccine with Montanide ISA 51 adjuvant in end-stage cervical cancer patients and showed that the vaccines were well tolerated, and elicited a broad interferon-γ-associated T-cell response in patients. They also conducted a study involving the vaccination of 11 HPV-16+ VIN grade III patients with the same long peptide vaccine and adjuvant, and a complete clinical immune response was seen in 4 of 11 patients.[93] Furthermore, the same vaccine regimen was also tested in patients with stage 1B1 HPV-16+ cervical cancer. The result of the trial showed increased HPV-16-specific CD4+ and CD8+ T-cell responses to a broad array of epitopes in all six patients.[31] A phase II trial to evaluate the effectiveness of a HPV-16 E6/E7 peptide-based vaccine in patients with metastatic or advanced cervical cancer is currently undergoing investigation.[32] Overall, the results from these early-phase clinical trials have generated a significant enthusiasm on therapeutic HPV E6/E7 long-peptide vaccines.

1.2.2 Protein-Based Vaccines

Protein-based vaccines, like peptide vaccines, are relatively safe compared with live vector-based vaccines. Furthermore, protein-based vaccines can circumvent the MHC specificity limitation associated with peptide vaccines. Since protein antigens can be processed in DCs, which contain all the possible human leukocyte antigen (HLA) epitopes of an antigen, this approach precludes the need to determine the HLA haplotype of prospective patients. The low immunogenicity of protein-based vaccines is a major drawback to its development; thus, strategies employing adjuvants and fusion with immunostimulatory molecules are often used to overcome this problem. Another concern for the development of protein-based vaccines is the limited efficacy of generating CTL responses, since they are often administered exogenously.

Adjuvants and fusion of immunostimulatory proteins have been used to increase the immunogenicity and CTL responses of HPV protein-based vaccines. For example, adjuvants such as the liposome-polycationic-DNA (LPD) adjuvant[94] and saponin-based adjuvant ISCOMATRIX®[95] have been shown to improve CTL responses of HPV protein-based vaccines. Furthermore, fusions of HPV antigen with molecules that can target the antigens to antigen-presenting cells (APCs) have been shown to increase the antigen uptake and presentation efficiency. Examples of such a strategy include fusion of HPV-16 E7 with *Bordetella pertussis* adenylyl cyclase (CyaA), a protein that targets APCs through specific interaction with integrin,[96] or fusion of HPV-16 E7 with the truncated bacterial exotoxin *Pseudomonas aeruginosa* exotoxin A, which facilitates translocation of protein to enhance MHC class I presentation.[97] Another important immunostimulatory molecule capable of enhancing CTL responses is heat shock protein (HSP) derived from *Mycobacteria*.[98,99]

Several HPV protein-based vaccines have been tested in clinical trials.[33-40] For example, a HPV fusion protein composed of HPV-6 L2 and E7 (TA-GW) has been tested in 42 healthy male volunteers[33] as well as 27 patients with genital warts.[34] TA-GW has been shown to be well tolerated in these clinical trials and was effective in generating antigen-specific T-cell responses in 19 patients and clearing HPV-associated genital warts in 5 patients. Another protein-based vaccine is TA-CIN, which utilizes a fusion protein comprised of HPV-16 L2, E6, and E7 antigens. It has been tested in 40 healthy volunteers and has shown no serious adverse effects. The vaccination with TA-CIN was also shown to induce antibody responses against L2 in all patients and T-cell immunity against HPV-16 E6 and E7 oncoproteins in 8 of 11 healthy patients receiving the highest dose.[35] In another early phase clinical trial, a vaccine (PD-E7) created from mutated HPV-16 E7 fused with a fragment of *Haemophilus influenzae* protein D, formulated in an adjuvant system containing Monophosphoryl Lipid A, QS-21 saponin adjuvant and oil-in-water emulsion (GlaxoSmithKline AS02B adjuvant), was shown to induce significant E7-specific CD8+ T-cell responses in patients with cervical intraepithelial neoplasia (CIN) 1 or CIN 3 lesions.[36] Furthermore, a vaccine comprised of HPV-16 E6/E7 fusion protein mixed with ISCOMATRIX® adjuvant was shown to be well tolerated and immunogenic, and showed significantly enhanced E6- and

E7-specific CD8+ T-cell responses in patients compared with those observed in placebo recipients.[37] Additionally, a fusion protein vaccine comprised of HPV-16 E7 and *M. bovis* HSP65 (HSPE7) was well tolerated in patients with high-grade anal intraepithelial neoplasia (AIN)[38] as well as in patients with CIN 3.[39,40] In a clinical study evaluating HSPE7 in patients with CIN 3, 13 of 58 patients showed complete pathologic responses, and 32 of 58 patients had partial responses, defined as colposcopic lesion regression of >50% in size.[39] However, it is not clear whether the response was due to natural regression rather than treatment effects. Clinical trials are ongoing in patients with CIN 3[41] and atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesions.[42] Recently, Nventa Biopharmaceuticals (bought by Akela Pharma) reported that the potency of HSPE7 could be further enhanced with the adjuvant Poly-ICLC, which serves as a foundation for the pursuit of phase I clinical trials of HSPE7 adjuvanted with Poly-ICLC.[43] More studies are needed to better define the clinical outcomes of the vaccine.

1.3 Nucleic Acid-Based Vaccines

1.3.1 DNA-Based Vaccines

DNA vaccines are attractive candidates for therapeutic HPV vaccines. DNA-based vaccines are stable, easy to produce, and can lead to sustained cellular gene expression compared with RNA or protein-based vaccines. Unlike the live vector-based vaccines, DNA vaccines do not lead to the generation of neutralizing antibodies and accordingly have the capacity for repeated administration. However, an important limitation of DNA vaccines is their limited potency, since they lack the intrinsic ability to amplify and spread *in vivo*. Therefore, it is important to consider strategies to improve DNA vaccine potency.

Strategies to Enhance DNA Vaccine Potency

It is now clear that DCs serve as a central player for DNA vaccine development because DCs are the most important professional APCs capable of priming naive T cells. The following section addresses the major directions and strategies used in enhancing DNA vaccine potency through modifications of DCs *in vivo* (for review, see Hung and Wu,[100] and Tsen et al.[101]). Table III summarizes the various strategies that have been developed to enhance the potency of therapeutic DNA vaccines for HPV.

Strategies to Increase the Number of Antigen-Expressing/Loaded Dendritic Cells (DCs)

The identification of efficient methods to deliver DNA directly into DCs may increase the number of antigen-expressing DCs. Intradermal administration of DNA vaccines via gene gun represents a potentially efficient way of delivering these vaccines to DCs. DNA-coated gold particles delivered by gene gun can efficiently deliver DNA to Langerhans cells, which are immature DCs present in the epidermis of the skin. The DNA-transfected Langerhans cells express the antigens encoded by DNA vaccines and become mature. Then, the antigen-expressing DCs migrate to the draining lymph nodes, where they prime naive T cells. In a head-to-head comparison study of DNA vaccines administrated by different methods, gene gun delivery required the smallest dose to generate similar responses compared with other methods such as biojector and intramuscular injection with syringe.[102] More recently, the feasibility of gene gun delivery of noncarrier naked DNA under a low-pressure system has been demonstrated. Noncarrier naked therapeutic HPV DNA vaccination was shown to result in significantly less local skin damage than gold particle-coated DNA vaccination, enhanced HPV antigen-specific T-cell immunity and antibody responses, and antitumor effects comparable with gold particle-coated therapeutic HPV DNA vaccination.[103]

Another strategy to increase the number of antigen-loaded DCs is the employment of intramuscular injection using electroporation. Usage of electroporation significantly enhances the uptake of DNA vaccines by muscle cells, resulting in more muscle cells expressing the antigen encoded by DNA vaccines. With an increased amount of antigen released by muscle cells, more DCs may be able to uptake and process the released antigens to activate antigen-specific T cells. It has recently been reported that intramuscular injection of HPV DNA vaccine in conjunction with electroporation could elicit potent HPV antigen-specific CTL responses.[104,105]

Other strategies used to increase the population of antigen-bearing DCs include the fusion of HPV antigens with molecules that are capable of concentrating and targeting the antigens to the DCs. Such molecules include FMS-like tyrosine kinase 3 (FLT3) ligands, which bind to FLT3 receptors on DCs,[106] and HSPs, which bind with scavenger receptors (e.g. CD91) on DCs.[102,107,108]

DNA vaccines do not spread beyond cells that are initially transfected. Increasing the spread of antigen encoded by a DNA vaccine can increase antigen loading by DCs. This has been done by linking antigen to proteins capable of intercellular transport. VP22 is a herpes simplex virus type 1 (HSV-1) microtubule binding protein. DNA encoding HPV-16 E7 fused to HSV-1 VP22 has been shown to enhance E7-specific CD8+ T-cell immune responses *in vivo* and generate stronger antitumor immune responses.[109] Strong antitumor responses have also been found using Marek's disease virus type 1 VP22

Table III. Strategies to enhance the potency of therapeutic human papillomavirus (HPV) DNA vaccines

Approaches	Strategies	Examples	References
Increasing number of antigen-expressing DCs	Route of administration	Gene gun	102,103
		Electroporation	104,105
	DC targeting	FLT3 ligands	106
		Heat shock proteins	102,107,108
	Intercellular antigen spreading	Linkage of Ag to intercellular trafficking proteins (HSV-1 VP22, MVP22)	109
Improving antigen expression, processing, and presentation in DCs	Enhance antigen expression in DC	Codon optimization	110-113
		Usage of demethylation agents	114
	Enhance antigen processing in DC	Link Ag with ER-targeting molecules	115
		Link Ag with molecules that facilitate proteasome degradation	116
	Enhance antigen presentation in DC	Link Ag to MHC class I-targeting proteins and protein domains	106,117-124
		Constant presentation of Ag through MHC class I via SCT technology	125,126
		Fuse antigen with MHC class II-targeting protein	127,128
		Improved Ag presentation through MHC class II via Ii-PADRE	129,130
Enhancing DC function and interaction with T cells	Prolong DC survival	Use of DNA encoding anti-apoptotic proteins or growth factors	131,132
		siRNA targeting pro-apoptotic proteins	133
	Prevent apoptosis of activated T cells	shRNA targeting pro-apoptotic FasL	134
	Enhance expression of stimulatory cytokines by DCs	Co-administration with immunostimulatory cytokines or adjuvants	135-139

Ag = antigen; **DC** = dendritic cell; **ER** = endoplasmic reticulum; **FasL** = Fas ligand; **FLT3** = FMS-like tyrosine kinase 3; **HSV-1** = herpes simplex virus type 1; **Ii** = invariant chain; **MHC** = major histocompatibility complex; **MVP22** = Marek's disease type 1 VP22; **PADRE** = Pan-DR binding T helper epitope; **SCT** = single chain trimer; **shRNA** = small hairpin RNA; **siRNA** = small interfering RNA.

(MVP22).[140] This strategy has also been applied to a naked Sindbis RNA replicon vector-based vaccine and was found to generate significant antitumor effects.[141] Taken together, efficacious administration routes, and employment of molecules that target HPV antigen to DCs and molecules that increase the intercellular spread of antigen encoded by DNA vaccines, are strategies that have been shown in preclinical models to enhance antigen expression by DCs, resulting in improvement of therapeutic HPV vaccine potency.

Strategies to Improve Antigen Expression, Processing and Presentation in DCs

One of the strategies to increase antigen expression in DCs is codon optimization, which eliminates codons not frequently used by the specific host and replaces them with more commonly recognized codons. This strategy can be used in both naturally occurring and recombinant gene sequences. Codon optimization has been shown to be effective in boosting the CTL response induced by HPV DNA vaccines.[110-113] Another strategy to increase antigen expression by DNA vaccines is the use of demethylation agents. It is known that DNA methylation leads to silencing of the genes that would affect the expression of the encoded antigen in a DNA vaccine. Recently, Lu et al.[114] demonstrated that administration of CRT/E7 DNA vaccine, combined with the demethylating agent 5-aza-2′-deoxycytidine (DAC), leads to upregulation of CRT/E7 expression, thus enhancing DNA vaccine potency.

DCs must efficiently process the antigens and present them through the MHC class I pathway to generate antigen-specific CD8+ T-cell responses. Researchers have also attempted to link HPV E7 antigens with molecules that target the endoplasmic reticulum[115] or facilitate proteasome degradation.[116] For example, DNA vaccines encoding E6/E7 antigen linked to

various MHC class I-targeting proteins and protein domains, includes *M. tuberculosis* HSP70,[117] HSP60,[118] CRT,[119-121] Gp96,[122] γ-tubulin,[123] the extracellular domain of FLT3-ligand,[106] and the translocation domain of *P. aeruginosa* exotoxin A.[124] These strategies have been shown to significantly improve MHC class I presentation of E6/E7 antigens and result in potent E6/E7 antigen-specific CTL responses generated by therapeutic HPV DNA vaccines.

Another strategy to enhance the antigen presentation by DCs involves the generation of a DNA construct encoding a fusion protein that links an antigenic peptide to the β2-microglobulin and MHC class I heavy chain, called single chain trimer (SCT) technology.[125,126] The expression of the encoded fusion protein by the DNA-transfected DCs will lead to a constant presentation of the antigenic peptide by MHC class I molecule. It has been shown in mice that vaccination with a DNA construct encoding a SCT, composed of HPV-16 E6 antigenic peptide fused with β2-microglobulin and H-2K^b MHC class I heavy chain, could generate significantly increased E6-specific CD8+ T-cell response compared with vaccination with DNA encoding HPV-16 E6 antigen.[125]

Significant endeavors have been made to improve the MHC class II presentation of antigens by using DNA encoding antigen fused with intracellular targeting protein. It has been shown that linkage of E7 antigen to a signal peptide for the endoplasmic reticulum (Sig) and the sorting signal of the lysosomal-associated membrane protein 1 (LAMP-1) can change the location of E7 from cytoplasm/nucleus to endosomal/lysosomal compartments, an important location for MHC class II presentation, and result in the enhancement of MHC class II presentation of E7 to CD4+ T helper cells.[127] Vaccination of the DNA encoding the chimeric Sig/E7/LAMP-1 protein has led to increased E7-specific CD4+ T-cell responses and antitumor effects against E7-expressing tumor in vaccinated mice.[128]

The MHC class II-associated invariant chain (Ii) has also been employed to improve antigen presentation through MHC class II pathway to enhance DNA vaccine potency. By substituting the class II-associated invariant chain peptide (CLIP) region of the Ii with a T helper epitope such as Pan-DR (PADRE) [Ii-PADRE], the epitope can thus be presented by the MHC class II pathway efficiently. Mice vaccinated with a DNA vaccine encoding Ii-PADRE can generate significant PADRE-specific CD4+ T-cell responses. Furthermore, co-administration of DNA encoding E7 and DNA encoding Ii-PADRE was shown to elicit potent E7-specific CD8+ T-cell responses.[129] The activated PADRE-specific CD4+ T helper cells, which can secret IL-2, enhances the E7-specific CD8+ T-cell immune responses generated by DNA vaccination.[130]

Strategies to Enhance DC Function and Interaction with T Cells

In order to improve DC interaction with T cells, it is important to consider the following: (i) prolonging the life of DCs; (ii) preventing apoptosis of activated T cells; and (iii) increasing expression of cytokines by DCs.

DNA vaccines employing strategies to prolong DC life were shown to further improve antigen-specific CTL responses.[142,143] DNA encoding anti-apoptotic proteins can be co-delivered with the vaccine to increase DC resistance to CTL-mediated killing. In previous studies, co-delivery of E7 DNA with DNA encoding BCL-xL, BCL2, XIAP, or dominant-negative caspases have been shown to enhance E7-specific CD8+ T-cell responses in mice.[131] However, the introduction of anti-apoptotic proteins might raise the concern for oncogenicity. An alternative solution to the problem is to apply the RNA interference (RNAi) technology to knockdown the pro-apoptotic proteins. For example, Kim et al.[133] have demonstrated that the co-administration of E7 DNA vaccines with small interfering RNA (siRNA) targeting BAK and BAX was effective in enhancing DC resistance to apoptosis and enhanced E7-specific CD8+ T-cell immune responses in the vaccinated mice. Recently, connective tissue growth factor (CTGF), important for cell survival, has also been used to prolong DC life. It has been shown that DNA encoding CTGF linked to E7 antigen can prolong the survival of DCs and generate potent antitumor responses.[132]

Another strategy to improve DCs and T cells interaction is to prevent the apoptosis of activated T cells. Fas ligand (FasL) is a key pro-apoptotic signaling protein expressed on the surface of DCs and can bind to its cognate ligand, Fas, on T cells. The binding of Fas on T cells to FasL on DCs can lead to T-cell apoptosis. Recently, Huang et al.[134] have shown that co-administration of E7 DNA and DNA encoding small hairpin RNA (shRNA) targeting FasL can generate significant E7-specific CTL responses. The downregulation of the FasL on DCs by RNAi may improve the survival of the activated T cells and result in increased antigen-specific CTL responses.

Another approach to improve the interaction between T cells and DCs is to enhance the expression of relevant cytokines by DCs. Co-administration of DNA vaccines encoding HPV antigens with DNA encoding GM-CSF,[135] IL-2,[136] or IL-12[137] have been shown to improve HPV antigen-specific immune responses. Moreover, HPV-16 E7-based DNA vaccines with DNA encoding sequence-optimized adjuvants such as IL-2 and IL-12 have also been shown to enhance E7-specific CTL responses.[138] Furthermore, HPV DNA vaccine encoding E7 linked to IL-6 has been shown to increase E7-specific T-cell immunities, anti-E7 antibody responses, and antitumor effects against E7-expressing tumors.[139]

Several DNA vaccines for HPV have been investigated in clinical trials. A microencapsulated DNA vaccine encoding multiple HLA-A2-restricted HPV-16 E7 epitopes (ZYC-101) has been tested in patients with CIN 2/3[45] and in patients with high-grade AIN.[44] The vaccine was well tolerated in both trials and shown to enhance E7-specific immune responses in some of the patients. A newer version of the DNA vaccine, ZYC-101a, which encodes HPV-16 and HPV-18 E6- and E7-derived epitopes, has been used in a phase II clinical trial in patients with CIN 2/3 lesions. This DNA vaccine has been shown to promote the resolution of CIN 2/3 in most (70%) of the patients younger than 25 years, compared with the placebo group of the same age.[46] A phase II/III trial is currently ongoing to evaluate the vaccine in patients with CIN 2/3.[47] At the Johns Hopkins Hospital, a phase I trial using a DNA vaccine encoding modified HPV-16 E7 DNA (with abolished Rb-binding site) linked with *M. tuberculosis* HSP70 [Sig/E7(detox)/HSP70] was tested in patients with CIN 2/3 lesions. The results of the trial showed that the vaccine was well tolerated by all the patients, and among the patients who received the maximum dosage of vaccine, some showed detectable E7-specific CD8+ T-cell immune response. In addition, complete histological regression of the lesions was observed in three of nine individuals in the highest-dose cohort.[48] The same DNA vaccine [Sig/E7(detox)/HSP70] has also been tested in HPV-16+ patients with advanced head and neck squamous cell carcinoma (M. Gillison and T.-C. Wu, personal communication). The same investigators also plan to initiate a phase I trial with a DNA vaccine encoding the modified HPV-16 E7 linked to CRT [CRT/E7(detox)] in patients with high grade intraepithelial cervical lesion using a clinical-grade gene gun device (C. Trimble, W. Huh, and T.-C. Wu, personal communication). More recently, a phase I clinical trial using DNA vaccine encoding HPV-16 and -18 modified E6 and E7 antigens (VGX-3100) via electroporation in patients with CIN 2 or 3 lesion was initiated and is ongoing.[49]

1.4 Naked RNA Replicon Vaccines

Naked RNA replicons for therapeutic HPV vaccine development have been tested in preclinical models. RNA replicons are RNA molecules that can replicate in a self-limiting fashion within the transfected cell. They may be derived from alphavirus, such as Semliki Forest virus,[144,145] Sindbis virus,[146,147] or Venezuelan Equine Encephalitis.[85,148] The RNA replicon vaccine can be administered in the form of RNA or DNA, which transcribes into the RNA replicons. One obvious advantage of the RNA replicon vaccine is its ability to self-replicate in a variety of cells, which can help sustain the cellular antigen expression, and thus enable them to produce more protein of interest than conventional DNA vaccines. Since most RNA replicon vectors have been modified to lack the viral structural genes, they do not form viral particles. Thus, RNA replicon vaccines may be repeatedly administered in patients without the generation of neutralizing antibodies against viral capsid protein. In addition, RNA replicons can bypass the possibility of chromosomal integration and cellular transformation that is associated with DNA vaccines. However, RNA is generally less stable than DNA, and is easily degraded in the injected host.

Attempts have been made to combine the benefits of RNA replicons and DNA vaccine by making a DNA-launched RNA replicon vaccine, so-called 'suicidal' DNA. This suicidal DNA can be transcribed into RNA replicons and provide a stable way to express encoded antigens. The suicidal DNA is more stable and easier to prepare than naked RNA replicons. Because uptake of the suicidal DNA vector into cells will eventually lead to apoptosis, there are no concerns of integration or transformation in the transfected cells. A suicidal DNA vector has been used for therapeutic HPV vaccine development in preclinical models and has been shown to generate significant HPV antigen-specific CD8+ T cell-mediated immune responses and antitumor effects.[149] Because the delivery of suicidal DNA vector by gene gun will make transfected cells such as DCs undergo apoptosis, leading to poor immunogenicity, Kim et al.[150] have generated a suicidal DNA vector, pSCA1, encoding E7 fused with BCL-xL, an antiapoptotic protein of the BCL2 family, to enhance the survival of APCs. These vaccines have shown to generate higher E7-specific CD8+ T-cell immune responses and better antitumor effects than suicidal DNA vector encoding wild type E7 alone in preclinical models.

Another strategy to alleviate the concern of apoptosis associated with the RNA replicon system involves the use of a flavivirus called Kunjin (KUN) to deliver antigens of interest to the cells. The key advantage of the KUN replicon vector is that it does not induce apoptosis in the transfected cells, thus enabling a more prolonged antigen presentation time by the transfected DCs compared with other RNA replicon vectors.[151] Vaccination of mice with DNA-launched KUN replicons encoding HPV-16 E7 epitopes generated E7-specific T-cell responses and protected vaccinated mice against tumor challenge of E7-expressing murine tumors.[152] Despite the general success of naked RNA replicon vaccines in preclinical models, they have not yet been tested in clinical trials.

1.5 Whole Cell Vaccines

1.5.1 DC-Based Vaccines

The increasing understanding of DC biology as well as the improved methods for preparing DCs *ex vivo* has paved the way

for DC-based vaccines. DCs can serve as natural adjuvants in antigen-specific cancer immunotherapy (for review, see Santin et al.[153]). A recent phase III clinical trial study using the DC-based cell vaccine (sipuleucel-T; Provenge®) in patients with advanced prostate cancer has shown encouraging results with improving overall patient survival compared with placebo.[154] The result has generated great enthusiasm for DC-based vaccines.

Although DC-based vaccines may seem promising, there are several serious limitations. The use of autologous DCs for individualized therapy will limit the large-scale production of the vaccine and will be technically demanding. Since culturing techniques will also affect the quality of the vaccines generated, it will be challenging to establish standard criteria for the preparation of DC-based vaccines. Furthermore, the route of administration is critical for the success of the vaccination, because it is essential for the DCs to target the T cells in the lymphoid organs to generate an effective immune response.

Nevertheless, DC-based vaccines have been used for HPV therapeutic vaccine development, and various methods have been used to prepare DCs for therapeutic HPV vaccines, including the usage of vectors,[155,156] pulsing DCs with proteins,[157] peptides,[158,159] or tumor cell lysates,[160] or transfecting DCs with DNA[161] or RNA.[162] Several strategies have been used to improve the DC-based vaccine. For example, one approach is to transfect DCs with siRNAs targeting key pro-apoptotic molecules such as BAK, BAX, and BIM to avoid T-cell-mediated apoptosis of DCs. The prolonged life of DC will lead to improved DC and T-cell interaction, and result in enhancement of T-cell priming. Vaccination with E7-loaded DCs transfected with siRNA targeting BAK and BAX has been shown to generate improved E7-specific immune responses and antitumor effects in mice.[158] More recently, vaccination with E7-presenting DCs transfected with siRNA targeting BIM was capable of generating a strong E7-specific CTL response and a marked therapeutic effect in vaccinated mice.[159]

DC-based vaccines have been tested in patients with HPV-associated cervical cancer. In a case report, a patient with advanced metastatic cervical cancer was treated with DCs loaded with HPV-18 E7 antigens. Although the vaccine did not induce complete remission in the patient, no significant adverse effects were observed.[50] In another clinical study, autologous DCs pulsed with HPV-16 or HPV-18 E7 recombinant protein were tested in 15 patients with late-stage cervical cancer. The result showed no local or systemic adverse effects, and E7-specific T-cell responses were observed in 4 of 11 patients.[51] Another clinical study using DCs pulsed with HPV-16 or HPV-18 E7 proteins was performed in four patients with advanced refractory cervical cancers. Elevated E7-specific CD4+ T-cell immune responses were observed in two of four patients, and E7-specific CD8+ T-cell immune responses were detected in all four patients.[52] In another clinical study, vaccination using autologous DCs pulsed with recombinant HPV-16/18 E7 antigens and keyhole limpet hemocyanin, an immunological tracer molecule, was shown to be well tolerated in stage IB or IIA cervical cancer patients and generated E7-specific CD8+ T-cell immune responses in eight of ten patients, and CD4+ T cell and antibody responses in all patients.[53] A pilot study using DC-based vaccine (HPV-16 E7 peptide-pulsed autologous DCs) for patients with recurrent cervical cancer is currently underway.[54]

1.5.2 Tumor Cell-Based Vaccines

Tumor cell-based vaccines are another approach to whole-cell vaccines. Tumor cells can be isolated and manipulated to express immunomodulatory proteins *ex vivo* to enhance their immunogenicity. Cytokine genes such as IL-2,[163] IL-12,[164,165] and GM-CSF[165,166] have been used in HPV-transformed tumor cell-based vaccines.

Some tumor cell-based vaccines have been tested in preclinical studies. Vaccination of mice with GM-CSF-expressing E7-positive tumor cells has been shown to lead to increased E7-specific CTL response and potent antitumor immune response against E7-expressing tumors.[166] Although tumor cell-based vaccines have been used in clinical trials for colorectal carcinoma, renal cell carcinoma, and melanoma, they have not been tested in HPV-associated malignancies in patients (for review, see de Gruijl et al.[167]). One potential concern with using tumor cell-based vaccines is the possibility of introducing new cancers to the patient. On the other hand, the key advantage of using tumor cell-based vaccines is the convenience that tumor antigens do not have to be well defined. In addition, potentially more tumor antigens may be covered with this approach. Because cervical cancer has well known tumor-specific antigens, E6 and E7, most studies have focused on HPV antigen-specific cancer immunotherapy.

2. Combinational Approaches

2.1 Prime-Boost Regimen for Therapeutic HPV Vaccines

The availability of different forms of therapeutic HPV vaccines creates opportunities for prime-boost regimens to further enhance therapeutic HPV vaccine potency. For example, previous studies have shown that priming with a HPV-16 E6/E7 DNA vaccine followed by boosting with recombinant vaccinia[168] or adenovirus[169] or with the HPV-16 E6/E7 expressing tumor cell-based vaccine[170] elicited greater

HPV antigen-specific CD8+ T-cell immune responses in vaccinated mice than vaccination with DNA vaccine, viral vector vaccine or tumor cell-based vaccine alone. In another prime-boost study, mice first primed with a Sindbis virus RNA replicon containing HPV-16 E7 linked to *M. tuberculosis* HSP70 (E7/HSP70) were boosted with a recombinant vaccinia virus encoding E7/HSP70. Significantly increased E7-specific CTL responses were observed in vaccinated mice.[171] The prime-boost strategy has also been proven successful in a HPV-16 E7 protein prime and vaccinia boost regimen.[172] More recently, it was demonstrated that a prime-boost regimen of heterologous vaccination with Venezuelan equine encephalitis virus replicon particles encoding HPV E6/E7 antigen and recombinant vesicular stomatitis virus encoding HPV E6/E7 antigen was dramatically more immunogenic than homologous vaccination with either vector alone in both mouse and monkey models.[173]

Some of the prime-boost regimens have been evaluated in therapeutic HPV vaccine clinical trials.[55-57] For example, in a phase II clinical trial, a HPV protein-based vaccine, TA-CIN (HPV-16 L2/E6/E7 fusion protein), was used for priming and a recombinant vaccinia virus encoding HPV-16/18, E6/E7 fusion protein (TA-HPV) was used for boosting in 29 patients with anogenital intraepithelial neoplasia. No serious adverse effects were observed; in addition, 5 of 29 patients showed increased HPV-16-specific T cell-mediated immune responses. However, the results did not show significant advantage over single TA-HPV vaccination.[56] In another prime-boost regimen, ten patients with HPV-16+ high-grade VIN were primed with TA-HPV and boosted with TA-CIN. Among all of the patients, nine patients developed HPV-16-specific T-cell responses, and three showed significant reduction in the size of the lesion. However, the result did not show direct correlation between clinical and immunological responses.[57] More recently, a clinical trial using pNGVL4a/Sig/E7(detox)/HSP70 DNA prime followed by TA-HPV boost is currently underway in patients with CIN 2/3 lesions, evaluating whether or not the topical application of imiquimod can further enhance prime-boost administration.[58]

2.2 Combination of Therapeutic HPV Vaccines with Immunomodulatory Agents

It is now clear that an effective immune therapy should consider modulation of the tumor microenvironment. There are many factors within the tumor microenvironment that may hinder the success of effective immune therapies. For example, T regulatory cells can release immune suppressive cytokines such as IL-10[174] and transforming growth factor-β,[175] which can paralyze T-cell function. The depletion of T regulatory cells in the tumor microenvironment has been shown to significantly enhance therapeutic HPV vaccine potency.[176] Other factors contributing to tumor immune suppression in tumor microenvironment include B7 homolog-1 (B7-H1),[177] signal transducer and activator of transcription 3 (STAT3) [for review, see Yu et al.[178]] and MHC class I polypeptide-related sequence (MIC)-A and -B,[179] indoleamine 2,3-dioxygenase (IDO) enzyme,[180] and galectin-1.[181] These factors may serve as potential targets for immune modulation to enhance therapeutic HPV vaccine potency (for review, see Kim et al.[182]).

2.3 Combination of Therapeutic HPV Vaccines with Other Therapeutic Modalities

Therapeutic HPV vaccines may potentially be combined with other therapeutic modalities such as chemotherapy, radiation therapy, or other therapeutic agents to augment the therapeutic vaccine effects.[114,183-188] Several chemotherapies and radiotherapies have been shown to enhance the potency of therapeutic HPV vaccines. For example, Chuang et al.[188] showed that combination of apigenin, a chemotherapeutic agent that is abundantly present in common fruits and vegetables and possesses anti-carcinogenic properties (for review, see Patel et al.[189]), with therapeutic HPV DNA vaccine could improve therapeutic HPV vaccine potency. Treatment with apigenin led to apoptotic tumor cell death *in vitro* in a dose-dependent manner and rendered the E7-expressing tumor cells more susceptible to lysis by E7-specific cytotoxic CD8+ T cells. Furthermore, treatment of mice bearing E7-expressing tumors with apigenin combined with therapeutic HPV DNA vaccine generated enhanced E7-specific CD8+ T-cell responses, leading to potent therapeutic antitumor effects against the tumors.[188] Likewise, death receptor (DR5)-specific antibodies,[190] and the proteasome inhibitor bortezomib,[186] have also been shown to improve therapeutic HPV DNA vaccine potency.

More recently, low-dose radiotherapy has been combined with therapeutic HPV DNA vaccine for the control of E7-expressing tumors in a preclinical model.[187] Treatment with low-dose radiotherapy rendered the TC-1 tumor cells more susceptible to lysis by E7-specific CTLs, and significantly enhanced therapeutic antitumor effects generated by HPV DNA vaccine.[187]

3. Concluding Remarks

Although preventive HPV vaccines are now commercially available, it is expected that it will take decades before such

vaccines can generate an impact on the incidence of cervical cancer. Thus, it is important to continue to develop safe and effective therapeutic HPV vaccines in order to accelerate the control of cervical cancer. The impressive preclinical data for therapeutic HPV vaccine development have led to several early-phase clinical trials.

The control of advanced cervical cancer will most likely require the combination of therapeutic HPV vaccine with other therapeutic modalities. With the increasing discovery of new drugs (i.e. targeted therapeutic agents and chemotherapeutic agents), as well as the better understanding of tumor biology, we will have greater opportunities to combine these treatment modalities with therapeutic HPV vaccines in order to improve therapeutic effects against HPV-associated cervical cancer.

Acknowledgments

This review is not intended to be an encyclopedic one, and the authors apologize to those not cited. We gratefully acknowledge Dr Richard Roden for his critical review of the manuscript. The work is supported by the NCI SPORE in Cervical Cancer P50 CA098252 and NCI 1RO1 CA114425-01.

The authors have no conflicts of interest that are directly relevant to the content of this review.

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