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Preventive and Therapeutic Vaccines for Human Papillomavirus-Associated Cervical Cancers

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Abstract

'High risk' genotypes of the human papillomavirus (HPV), particularly HPV type 16, are the primary etiologic agent of cervical cancer. Thus, HPV-associated cervical malignancies might be prevented or treated by induction of the appropriate virus-specific immune responses in patients. Sexual transmission of HPV may be prevented by the generation of neutralizing antibodies that are specific for the virus capsid. In ongoing clinical trials, HPV virus-like particles (VLPs) show great promise as prophylactic HPV vaccines. Since the capsid proteins are not expressed at detectable levels by basal keratinocytes, therapeutic vaccines generally target other nonstructural viral antigens. Two HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are coexpressed in the majority of HPV-containing carcinomas. Therefore, therapeutic vaccines targeting these proteins may provide an opportunity to control HPV-associated malignancies. Various candidate therapeutic HPV vaccines are currently being tested whereby E6 and/or E7 are administered in live vectors, in peptides or protein, in nucleic acid form, as components of chimeric VLPs, or in cell-based vaccines. Encouraging results from experimental vaccination systems in animal models have led to several prophylactic and therapeutic vaccine clinical trials. Should they fulfill their promise, these vaccines may prevent HPV infection or control its potentially life-threatening consequences in humans.

Introduction

The Role of Human Papillomavirus in Cervical Cancers

Approximately 500,000 women worldwide develop cervical cancer yearly and it is the second leading cause of cancer death in women. Despite the availability of cervical cancer screening in the US, the incidence of invasive cervical cancer does not seem to be diminishing (SEER Cancer Statistic Review). Over the past 15 years, epidemiologic/virologic data have identified a clear and consis-

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tent association of human papillomavirus (HPV) infection with the development of cervical cancer. The evidence linking HPVs to cervical cancer comes from a wide variety of epidemiologic and laboratory studies. More than 99% of cervical cancers and their precursor lesions, squamous intraepithelial lesions (SIL), contain HPV DNA [144]. Furthermore, molecular, biochemical and cellular studies have unequivocally demonstrated that E6 and E7, two HPV gene products that are consistently expressed in precursor lesions (SIL) and cervical cancer, lead to malignant transformation of epithelial cells [for review, see 151]. E6 binds, inactivates and promotes the degradation of p53, while E7 binds and inactivates pRb [86, 112, 149]. Although over a hundred HPV genotypes have been identified, about 80% of cervical cancer is associated with four 'high risk' types of HPV (types 16, 18, 31, 45) and the remaining cases are associated with a dozen other oncogenic types [for review, see 124]. HPV-16 is present in approximately 50% of all cervical cancers [75] and is consequently the focus of many recent HPV vaccine developments.

Events in the Progression from HPV Infection to Cervical Cancer

Clinical, pathological and virologic studies have defined a progression of events in the development of cervical cancer [for review, see 55, 118]. The pathogenesis of cervical cancer is initiated by HPV infection of cervical epithelium during sexual intercourse. The majority of genital HPV infections are transient. However, a fraction of infections persist and initiate transformation events within cervical epithelium. The cervical epithelial changes associated with this initial set of events, pathologically classified as low-grade SIL (LSIL or CIN 1), are associated with continued viral replication and virus sheeding [for review, see 55, 118]. In a study of 241 cytologically normal women recruited in a sexually transmitted disease clinic, the cumulative incidence of high-grade SIL (HSIL or CIN 2–3) at 2 years was 28% in HPV-positive women compared to 3% in HPV-negative women [66]. The progression from HSIL to invasive cancer occurs at high frequency. In the majority of cases, progression is associated with conversion of the viral genome from an episomal form to an integrated form, along with deletion or inactivation of E2, a negative regulator of E6 and E7 expression [55]. Development of invasive cancer requires additional genetic events facilitated by E6- and E7-mediated inactivation of the genome guardians p53 and pRb, genomic instability and suppression of apoptosis [55].

Importance of Humoral Immune Responses in Preventing HPV Infections

Animal studies suggest that virus-neutralizing antibodies can protect the host from infection. Recombinant HPV virus-like particles (VLPs) are generated by overexpression of L1, the major capsid protein of HPV, which display neutralizing epitopes [64]. Immunization of animals with VLP protects against experimental infection with the homologous animal papillomavirus [12, 65, 127]. Indeed, passive transfer of sera from VLP-vaccinated mice to naïve mice is sufficient in generating protection, indicating that the protective effect is likely mediated by neutralizing antibodies [12, 127]. Experiments with heterologous VLPs have demonstrated no protective immune effect, indicating the specificity and non-cross-reactivity of VLPs. Furthermore, denatured VLPs demonstrated no protective immune effect, indicating that protection required intact VLPs with conformational epitopes [12]. The other late gene is L2, which encodes the minor capsid protein of HPV. Although various studies have demonstrated that vaccination with L2 fusion proteins prevent experimental papillomavirus infections [29, 59, 73], VLPs generate even higher titers of serum-neutralizing antibodies. Thus, VLPs may be more efficacious than bacterially expressed L2 as prophylactic vaccines. However, vaccination with L1/L2 VLP proved no more effective than L1 alone [12]. Nevertheless, the experimental data in animals suggests that both VLP and L2 represent promising prophylactic vaccines [77, 114].

Unfortunately, immunization with capsid proteins fails to generate significant therapeutic effects for established or breakthrough HPV infections that have escaped antibody-mediated neutralization. This is likely because the capsid genes are only expressed upon terminal differentiation in the upper strata of the epidermis, but not in basal keratinocytes. Preexisting HPV infection is highly prevalent and responsible for considerable morbidity and mortality. A different vaccination strategy is required to treat this infected population. Evidence suggests that cellular immunity, particularly antigen-specific T cell-mediated immunity, is required for treatment of established HPV infection [4]. Therefore, vaccines that induce cell-mediated immunity specific for nonstructural viral proteins are more likely to effect regression of established lesions or even malignant tumors.

Importance of Cell-Mediated Immune Responses in Controlling Established HPV Infections and HPV-Associated Neoplasms

Several lines of evidence suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms [for review, see 151]. First, the prevalence of HPV-related diseases (infections and neoplasms) is increased in transplant recipients [49] and human immunodeficiency virus-infected patients [69, 110], both of whom are known to have impaired cell-mediated immunity. Second, animals immunized with nonstructural viral proteins are protected from papillomavirus infection or the development of neoplasia. Immunization also facilitates the regression of existing lesions [11, 117]. Third, infiltrating CD4+ (T helper cells) and CD8+ (cytotoxic T cells) T cells have been observed in spontaneously regressing warts [129] and fourth, warts in patients who are on immunosuppressive therapy often disappear when treatment is discontinued [for review, see 4]. Therefore, effective therapeutic HPV vaccines should generate enhanced HPV-specific cell-mediated immune responses.

Importance of Dendritic Cells in Mediating Immune Responses

Dendritic cells (DCs) are potent professional antigen-presenting cells (APCs) specialized to prime helper and killer T cells in vivo [for review, see 16, 52, 122]. Increasing evidence suggests that professional APCs (which include DCs, macrophages, and B cells) are central players for mediating immunotherapy. DCs highly express MHC-I and MHC-II molecules, costimulatory molecules such as B7, and adhesion molecules including ICAM-1, ICAM-3 and LFA-3 which are involved in antigen-specific T cell activation. To effectively present antigens, DCs perform a series of coordinated tasks [102]. In the presence of maturation-inducing stimuli such as inflammatory cytokines or activation vis CD40 [142], DCs upregulate the expression of adhesion and costimulatory molecules and become more potent and differentiated stimulators of T cell-mediated immunity. DCs are equipped to capture antigens and present large numbers of immunogenic MHC-peptide complexes on their surface [15, 95]. In addition, DCs migrate to secondary lymphoid organs where they stimulate rare antigen-specific T cells [2]. Effective vaccines most likely require a strategy that targets tumor antigens to professional APCs, such as DCs, along with a means of enhancing MHC class I and/or class II presentation of the tumor antigen to activate antigen-specific T cells.

HPV Vaccine Development

The well-defined virologic, genetic and pathological progression of HPV – from initial infection to lesion formation to malignant tumor formation – and the limited number of well-defined foreign (viral) antigens provide a unique opportunity to evaluate intervention with antigen-specific immunotherapy at various stages of HPV infection and tumorigenesis. Conceptually, two different types of HPV vaccines can be designed: prophylactic (preventive) vaccines that prevent HPV infection and therapeutic (curative) vaccines that would induce regression of established HPV infection and its sequelae.

Preventive HPV Vaccines

Preventive HPV vaccine development has been complicated by the lack of animal models for the genital mucosatropic HPV types and by the difficulty in propagating the virus in culture. These difficulties have been partially overcome using cutaneous and mucosal animal papillomaviruses as models and by the development of VLPs and pseudovirion, yet these models do not adequately simulate sexual transmission. The papillomavirus major capsid protein L1 – when overexpressed in mammalian [47, 158], insect [64], yeast [109] or bacterial cells [5, 88] – spontaneously assembles to form VLPs that are devoid of the oncogenic viral genome. Parenteral injection of these VLPs elicits high titers of serum-neutralizing antibodies and protection from experimental challenge with infectious virus in several animal papillomavirus models [12, 65, 127]. Protection from experimental infection by cottontail rabbit papillomavirus or canine oral papillomavirus following passive transfer of IgG from immunized animals to naïve animals has been demonstrated in rabbits and dogs, respectively [12, 127]. The results from these protective vaccine studies indicate that humoral responses effect protection from experimental infection.

Although VLP vaccination provides immunity from experimental inoculation, it is unclear whether this extends to protection against natural transmission of genital HPV. To completely prevent sexual transmission of genital HPV infection, neutralizing antibodies must act at

mucosal surfaces, which are the natural site of infection. Antibodies both pass from plasma into genital secretions and are synthesized by local plasma cells [92, 132]. The plasma cell precursors that migrate to the genital tract are derived primarily from mucosal lymphoid tissues and predominantly secrete IgA. Induction of these cells requires direct immunization of the mucosa-associated lymphoid tissue and in several experimental systems, nasal instillation was found to be the most effective route of immunization to generate specific antibodies in genital secretions in mice and in monkeys [3, 107]. More recently, oral vaccination with HPV VLPs in mice has been shown to induce systemic virus-neutralizing antibodies, suggesting that the HPV VLPs may be antigenically stable in the environment of the gastrointestinal tract. These studies provide the possibility of vaccinating large populations with HPV VLP without using syringes [103].

Local, sustained production of secretory IgA and/or specific IgG is likely required for long-lasting sterilizing immunity. In previous studies, systemic immunization of mice with purified HPV VLPs induced no detectable mucosal IgA antibodies and low titers of IgG [3, 46]. Furthermore, low titers of VLP-specific IgG, and no IgA, were detected in cervicovaginal lavage of parenterally immunized monkeys [76]. Although these experiments in monkeys showed that transudated IgG alone partially neutralized HPV-11 in vitro, the mucosal antibody response was short-lived [76]. Nasal immunization using HPV-16 VLPs has been shown to induce significant and sustained titers of HPV-16-neutralizing antibodies in both serum and mucosal secretions of mice [3, 37, 88].

The promising results generated in preclinical animal studies have led to phase I/II clinical trials using the HPV-16 VLP vaccine delivered intramuscularly. No significant toxicity has been observed in these trials [Schiller, pers. commun.]. Furthermore, neutralizing antibodies were generated as a result of vaccination. Currently, phase III clinical trials using intramuscular administration of the HPV-16 VLP vaccine are being planned in Guanacaste, Costa Rica [Schiller and Hildesheim, pers. commun.].

Although VLP-based vaccines represent good candidate prophylactic HPV vaccines, several issues remain to be addressed. It is unclear whether sexual transmission is due to free viral particles or to particles still enclosed in detached squamous cells that might protect the virus from neutralizing antibody. Furthermore, the frequent and intimate nature of sexual contact implies a potentially large HPV inoculum, suggesting the potential for breakthrough infection that escapes antibody-mediated neutralization. On the basis of observations made in both humans and animals, it appears that HPV-specific antibodies are insufficient for clearing preexisting papillomavirus infection [7]. Finally, established HPV infection is highly prevalent in the population and responsible for significant morbidity. It is therefore important to consider therapeutic intervention.

Therapeutic HPV Vaccines

Therapeutic vaccines should induce specific cell-mediated immunity that prevents the development of lesions and eliminates preexisting lesions or even malignant tumors. Theoretically, the induced specific cellular immunity can directly target HPV viral products, HPV-induced cellular products, or a combination of both. However, since little is known about which cellular products serve as targets for specific cellular immunity, most of the experimental vaccination systems use carcinoma-associated HPV proteins, particularly E6 and E7, as targets for specific cellular immunity.

L1 and L2 capsid proteins are unlikely to be suitable targets for therapeutic vaccine development because these proteins are not detectably expressed in basal epithelial cells of benign lesions or in abnormal proliferative cells of premalignant and malignant lesions [125, 126]. Although a recent study indicated that vaccination with VLPs may generate a capsid protein-specific cytotoxic T lymphocyte (CTL) response [106], such a response against L1 or L1/L2 VLPs alone would not likely result in a significant therapeutic effect.

Since E6 and E7 are consistently expressed in most cervical cancers and their precursor lesions but absent from normal tissues, these viral oncoproteins represent promising targets for the development of antigen-specific therapeutic vaccines for HPV-associated cervical malignancies and their precursor lesions. While most tumor-specific antigens are derived from normal or mutated proteins, E6 and E7 are completely foreign viral proteins, and may therefore harbor more antigenic peptides/epitopes than a mutant cellular protein. Furthermore, since E6 and E7 are required for the induction and maintenance of the malignant phenotype of cancer cells [32], cervical cancer cells are unlikely to evade an immune response through antigen loss. Finally, studies in animal models suggest that vaccination targeting papillomavirus early proteins such as E7 can generate therapeutic as well as protective effects [14]. Therefore, E6 and E7 proteins represent good targets for developing antigen-specific immunotherapies or vaccines for cervical cancer.

Various forms of HPV vaccines, such as vector-based vaccines [8, 72, 84], peptide-based vaccines [39], protein-based vaccines [40, 68], DNA-based vaccines [19–21, 62, 120], chimeric VLP-based vaccines [44, 93, 111] and cell-based vaccines [23] have been described in experimental systems targeting HPV-16 E6 and/or E7 proteins. In most studies, researchers mainly focused on E7 because it is more abundantly expressed and better characterized immunologically. Furthermore, its sequence is more conserved than the E6 gene [155]. These experimental vaccines are summarized in table 1.

The following sections will describe each of the therapeutic vaccines outlined in table 1.

Table 1. Therapeutic HPV vaccines

Vector-based vaccines
Viral vectors: AdV, vV
Bacterial vectors: *L. monocytogenes, S. typhimurium*
Peptide-based vaccines
HLA-A.2 haplotype-specific vaccines
Adjuvants for peptide-based vaccine: ISCOMs, cytokine, polycation, HSP, lipopeptide
Protein-based vaccines
Adjuvants for protein vaccines: cytokines, HSP, GM-CSF linked to protein, CpG oligodeoxynucleotides
DNA vaccines
Route of DNA vaccines: intramuscular, intradermal via gene gun, intravenous or intranasal
Strategies to enhance DNA vaccine potency: adjuvants, cytokines, LAMP-1 targeting, HSP
Chimeric VLP-based vaccines
Cell-based vaccines
DC-based vaccines
DC pulsed with peptides/proteins
DC transduced with HPV E6 and/or E7 genes
Tumor cell-based vaccines: GM-CSF-transduced
Self-replicating RNA vaccines
RNA-launched RNA replicons
DNA-launched RNA replicons, 'suicidal' vectors
HPV pseudovirion vaccines

Live Vector-Based Vaccines

Vector-based vaccines can be used to express HPV E6 and/or E7 for the prevention and treatment of HPV-associated neoplasms. These vectors can be classified into the following categories: (1) viral vectors, such as adenovirus (AdV) and vaccinia virus (vV) and (2) bacterial vectors, such as *Listeria monocytogenes* and *Salmonella typhimurium.*

Viral Vectors

AdV Vector. Recombinant AdV are widely used vectors with a cloning capacity approximately 8 kb in size, allowing for the insertion of a relatively large gene. They can be prepared easily in high titer. AdV can transduce a wide range of cell types with remarkable transduction efficiency without integrating into the host genome, eliminating the safety concern of insertional mutagenesis. The major concern for immunization is the production of anti-AdV antibodies by the host, which may inhibit repeat vaccination and thereby compromise the therapeutic effect. However, a previous study demonstrated that preexisting immunity to AdV does not prevent the antitumor effect following intratumoral administration of an IL-12-expressing AdV vector [10]. According to various studies, recombinant AdV vectors encoding a tumor-specific antigen such as P815A [147], β-gal [24] or gp100 [156] can induce an antigen-specific CTL response and antitumor effect. Since AdV has a large cloning capacity, multiple CTL epitopes can be cloned to a single AdV. Protective antitumor immunity to multiple tumor antigens can be induced in a murine model by vaccination with recombinant AdV encoding multiple tumor-associated CTL epitopes, including those from HPV, in a string-of-beads fashion [137]. The advantages of AdV encoding multiple CTL epitopes derived from several tumor-specific antigens are that the risk of tumor immune escape by antigen loss or antigen mutation is relatively low and it largely eliminates the risk of transformation by recombinant vector carrying functional oncogenes.

VV Vector. VV, a member of the poxvirus family, can also be used to mediate the transfer of genes into host APCs. This strategy offers several appealing features including high efficiency of infection and high levels of recombinant gene expression [85]. Infection with recombinant vV and expression of the desired gene product occurs quickly. Furthermore, the vaccinia genome can accommodate large recombinant gene insertions. The availability of replication-deficient recombinant poxvirus, such as canarypox virus, has provided the opportunity to use this recombinant virus as a vector for gene transfer into host APCs. First, these constructs are likely to be extremely safe, since productive viral replication is restricted to avian species so that infection of mammalian cells fails to generate infectious viral particles [96]. Second, T cell responses against vaccinia antigens (present in most of the adult population immunized against smallpox) do not significantly cross-react with canarypox antigens [43], obviating the concern that preexisting immunity would preclude immunization with canarypox virus.

Finally, vV is a lytic virus and thus the chance of integration of vaccinia genome into the host genome is extremely small. These characteristics make vV a suitable vector for tumor vaccine. Several studies have shown that E6- and/or E7-specific immunotherapy using vaccinia vectors has generated strong CTL activity [9, 42] and an antitumor effect in preclinical studies [18, 61, 72, 84]. Furthermore, a recombinant vV encoding HPV-16 and HPV-18 E6/E7 has been used for a phase I clinical trial in cervical cancer patients [8]. No significant complications or environmental spread of vV was noted in these trials. Recently, vV has also been utilized to explore tumor vaccine strategies employing intracellular sorting signals. The increased understanding of intracellular pathways for antigen presentation has created the potential for designing novel strategies to enhance vaccine potency. For example, the endosomal and lysosomal compartments are associated with MHC-II processing and presentation. These compartments are characterized by the presence of a number of compartment-specific membrane proteins, including the lysosomal associated membrane protein (LAMP-1). In a previous study, Wu et al. [152] linked to sorting signals of LAMP-1 to the HPV-16 E7 antigen to create the Sig/E7/LAMP-1 chimera. They found that expression of this chimera in vitro and in vivo with a recombinant vV targeted E7 to endosomal and lysosomal compartments and enhanced MHC class II presentation to CD4+ T cells compared to vV expressing wild-type E7. Furthermore, the Sig/E7/LAMP-1 vV vaccine cured established tumors containing the E7 antigen in the murine model while wild-type E7 vV showed no effect on the established tumor [72]. These experiments demonstrate that modifications rerouting cytosolic antigen to the endosomal/lysosomal compartment can profoundly improve the in vivo therapeutic potency of recombinant vaccinia vaccines. The impressive results from this preclinical study have led to the planning of phase I/II clinical trials using intramuscular administration of attenuated Sig/E7/LAMP-1 Wyeth strain vaccinia at the Johns Hopkins Hospital, which will start in late 2000.

Bacterial Vectors

L. monocytogenes has recently emerged as an exciting prospect for use as a recombinant vaccine for human cancers due to its ability to generate both CD8+ and CD4+ immune responses and induce regression of established tumors expressing a model antigen. *L. monocytogenes* is a gram-positive intracellular bacterium that usually infects macrophages. When *L. monocytogenes* is phagocytosed by macrophages, it is taken up in a phagosome. However, unlike other intracellular bacteria, it escapes into the cytoplasm of the macrophage by secreting a factor, listeriolysin O, disrupting the phagosomal membrane [143]. Because of its presence in both endosomal compartments and cytoplasm, *L. monocytogenes* can deliver its antigens or carry foreign antigens into both the MHC-I and MHC-II pathways, inducing strong cellular immune responses. A recombinant *L. monocytogenes* secreting HPV-16 E7 has recently been shown to lead to regression of preexisting E7-expressing tumors using an E7-expressing murine tumor model, TC-1 [Paterson and Pardoll, pers. commun.]. Antigen-specific *L. monocytogenes* vaccines may also be administered orally without losing efficacy in mice [91]. These convincing preclinical results have led to the planning of phase I/II clinical trials on patients with advanced HPV-associated cervical cancer at the Johns Hopkins Hospital, presumably in 2001.

Mammalian expression vectors containing genes of interest can be transformed into attenuated bacteria, such as mutant strains of *Shigella, Escherichia coli,* or *Salmonella,* which can serve as bacterial carriers to deliver plasmid encoding genes of interest into APCs. Among these mutant bacteria, *Salmonella* have already been used as live vaccines in humans. The advantage of using *Salmonella* as a carrier is its natural route of infection, which even allows vaccination in an oral form [33]. After leaving the intestinal lumen, *Salmonella* migrates into the lymph nodes and the spleen, where it encounters macrophages and DCs. The attenuated *Salmonella* then release multiple copies of antigen-coding plasmid inside phagocytes, which leads to expression of the antigen and elicits strong immune responses. Alternatively, genes of interest such as antigen can be cloned into a prokaryotic expression vector, which is subsequently transformed into *Salmonella* and induced to express antigen of interest in bacteria. The bacteria can then be used as a carrier for the antigen in protein form as a vaccine. This approach has been used to deliver HPV-16 E7 [67] or E7 epitopes harbored in hepatitis B virus core antigen particles to generate E7-specific immune responses [74]. These results encourage the use of bacterial carrier systems such as *Salmonella* for delivering HPV vaccines.

Peptide-Based Vaccines

The characterization of many CTL-defined antigenic determinants has opened the possibility of developing antigen-targeted vaccines. Several HPV-16 E7-specific CTL epitopes have been characterized for the HLA-A.2 haplotype [63, 98]. Immunization with a peptide derived from the HPV-16 led to protection against a lethal dose of

HPV-16-transformed tumor cells [39]. In human studies, some HPV-associated cancer patients vaccinated with peptides derived from HPV-16 E7 showed CTL responses [123] and in one phase I/II study, no adverse side effects of peptide-based HPV vaccine were observed in patients [141].

The potency of HPV-16 E7 peptide-based vaccines can be further enhanced by the use of adjuvants such as immune stimulatory complexes (ISCOMs) [41] and immunostimulatory carriers (ISCAR) [135]. Cytokines such as IL-2 may boost the adjuvant effect in peptide-based vaccines according to a study using the gp100 melanoma-associated antigen [104]. Polycations, such as polylysine, can also act as adjuvants for peptide vaccines against cancer [116]. Another strategy involves the modification of CTL epitopes using lipid conjugation to form an immunogenic P3CSS lipopeptide vaccine [35, 113]. Two similar strategies have been adapted for the treatment of HPV-associated cancer including an E7 (aa 86–93) lipopeptide vaccine [123] and two P2-KGG lipid-tailed E6 and E7 peptide-based vaccines [108], both of which were shown to generate enhanced CTL responses. Finally, heat shock proteins (HSP) have been shown to be capable of delivering noncovalently bound peptide to MHC-I molecule and induce peptide-specific CTL responses [71, 121]. Mice immunized with autologous cancer-derived heat shock protein-peptide complexes resulted in retarded progression of the primary cancer, reduced metastatic load, and prolongation of life span in several murine tumor models [130].

Although vaccination with synthetic peptides corresponding to CTL epitopes can induce protective CTL responses and effective antitumor immunity in several murine tumor model systems, not all peptide-based vaccines generate CTL responses and tumor protection. For example, vaccination with a CTL epitope derived from the human adenovirus type 5 E1A region (Ad5E1A234–243) enhanced rather than inhibited the growth of Ad5E1A-expressing tumors [136, 138]. Interestingly, the same epitopes loaded onto DCs can generate protective immunity, indicating that it may not be the peptides per se, but rather the method of presenting the epitope to T cells that determines the outcome of immunization with peptide-based vaccines [Toes et al., pers. commun.]. It is, therefore, important to choose the appropriate adjuvants and route of administration for peptide-based vaccines in order to determine their immunizing or tolerizing properties in vivo before clinical use.

Protein-Based Vaccines

The application of peptide-based vaccines is limited by MHC restriction and the necessity to define specific CTL epitopes. Most CTL epitopes of HPV-16 E6 and/or E7 in patients with HLA other than HLA-A.2 remain undefined, making it difficult to use peptide-based vaccines in such situations. In addition, the preparation of peptide-based vaccines for use on a large scale is inefficient and laborious. These limitations can potentially be overcome by using protein-based vaccines. Protein-based vaccines can present all possible epitopes of a protein to the immune system, thus bypassing the MHC restriction. Furthermore, with a protein vaccine, dangerous side effects such as insertional gene activation and transformation, a potential concern with the use of recombinant viruses and DNA vaccines, are not an issue.

A growing number of modified exogenous protein antigens, including HPV-16 E7, have been shown to be capable of generating MHC-I-restricted CTL responses. Association of E7 protein with adjuvants, such as PROVAX [51], incomplete Freund's adjuvant [34], or saponin QS21, monophosphorylated lipid A, and oil-and-water emulsion [Pardoll et al., pers. commun.] is able to enhance E7-specific CTL activities. Studies have demonstrated that CTL priming is induced by injection of heat-aggregated HPV-16 E7 antigen [115]. Furthermore, the TA-GW fusion protein, which consists of HPV-6 L2 fused to E7 protein, has also been tested for clinical treatment of genital warts [68, 133].

Several strategies to increase the potency of protein-based strategies have yet to be tested in the HPV context. The fusion of antigen with heat shock proteins represents a strategy for enhancing CTL priming [128]. This strategy is currently being tested using *Mycobacterium tuberculosis*-derived HSP70 fused with HPV-16 E7 [Wu, pers. commun.]. Various studies have demonstrated that GM-CSF linked to an antigen can target the antigen to DC and other GM-CSF-responsive cells after the chimeric molecule binds to the GM-CSF receptor, generating enhanced immune responses in these cells [25, 131]. Immunostimulatory CpG oligodeoxynucleotides that contain unmethylated CpG motifs have also been shown to be able to enhance the potency of protein vaccines by inducing macrophages to secrete IL-12 and shift cytokine profiles to Th1-type immunity [30, 101]. CpG oligodeoxynucleotide is a promising alternative to complete Freund's adjuvant because it lacks significant toxicity [148], making it an attractive option for enhancing HPV protein-based vaccines.

Recent studies have elucidated a potential mechanism by which modified exogenous antigens are presented through the MHC-I pathway (also known as cross-priming). Initial studies have suggested that MHC-I presentation of exogenous antigen occurs by regurgitation of peptides generated in the phagosomal compartment to the cell exterior followed by binding to empty MHC-I molecules [50]. Another set of studies suggested a phagosome to cytosol transfer of antigens with ultimate cytosolic processing and TAP-dependent transport of peptides into the endoplasmic reticulum for binding to nascent MHC-I molecules [154]. A more recent study has demonstrated that a subset of MHC-I molecules is transported to the endosomal/lysosomal compartment, providing a pathway for class I loading that is shared with class II molecules [45].

DNA Vaccines

Naked DNA is an advantageous construct for vaccination because of its purity, simplicity of preparation and stability. In addition, DNA-based vaccines can be prepared inexpensively and rapidly in large scale. Since DNA allows for expression of antigen for a sustained period of time, the availability of antigen to be processed and presented as MHC-peptide complexes is likely more prolonged compared to peptide vaccines. Furthermore, the MHC restriction of peptide-based vaccines may be bypassed with approaches that directly transduce DNA coding for antigen inside APCs so that synthesized peptides can be presented by the patient's own HLA molecules. Since DNA vaccines targeting many different types of HPV can be mixed and effectively administered together, DNA vaccines provide an efficient method of treating a variety of HPV-associated infections and tumors. These advantages support the enthusiastic interest for the development of vaccines employing naked DNA. DNA vaccines can be administered to the host by intramuscular injection, intradermal injection via hypodermic needle or gene gun (a ballistic device for delivering DNA-coated gold particles into the epidermis), intravenous injection, or intranasal delivery [for review, see 36, 99].

Various studies have investigated the mechanisms involved following DNA vaccination via intramuscular injection or gene gun delivery. Following intramuscular injection, some myocytes are transfected by the DNA vaccine, causing them to produce protein and transfer antigen to bone marrow-derived professional APCs [134]. In an alternative hypothesis, injected DNA may move as free DNA through the blood to the spleen where professional APCs initiate responses [99]. Following gene gun delivery, epidermal Langerhans cells are transfected by the DNA vaccine and then serve as APCs. The DCs of the skin carry the antigens from the skin to the draining lymph nodes, where the antigen-loaded DCs activate the naïve T cells [31]. The method of DNA inoculation (gene gun vs. intramuscular injection) and the form of the DNA-expressed antigen (cell-associated vs. secreted) determine whether T cell help will be primarily type 1 or type 2 [for review, see 99].

The understanding of MHC-I and MHC-II antigen presentation processing and intracellular sorting pathways has created the opportunities of designing strategies that may enhance MHC-I and MHC-II antigen presentation and thereby lead to increased antigen-specific CD4+ and CD8+ T cell-mediated immune responses and tumor protection. LAMP-1 is an endosomal/lysosomal compartment-associated protein described earlier (see vV Vector) that is a viable strategy to enhance not only vaccina vaccine potency, but also DNA vaccine potency. The Sig/E7/LAMP-1 naked DNA vaccine has been shown to enhance MHC-I and MHC-II presentation of E7 to activate both E7-specific CD8+ and CD4+ T cells and a potent E7-specific antitumor effect compared to wild-type E7 DNA [21, 62]. Encouraging results from this preclinical study have led to the planning of phase I/II clinical trials at the Johns Hopkins Hospital using the Sig/E7/LAMP-1 naked DNA vaccine delivered intramuscularly, which presumably will start in late 2000. Another recent strategy has used *M. tuberculosis* HSP70 for the development of DNA vaccine to enhance E7-specific CD8+ T cell activities and antitumor effects [20]. HSPs are a family of chaperone proteins that facilitate the formation of complexes between MHC-I molecules and antigen, enhancing the immunogenicity of these HSP-peptide complexes. Immunization with HSP-peptide complexes isolated from tumor or virus-infected cells can induce potent antitumor or antiviral immunity. Chen et al. [20] demonstrated that E7-HSP70, a chimeric DNA vaccine attaching HSP70 gene to the 3′ end of an E7 gene, generated significantly enhanced E7-specific CTL responses and antitumor effects compared to constructs that only contained control plasmid, E7 alone, HSP alone, or E7 and HSP70 in an unfused mixture. While E7-HSP70 generates potent CD8+ T cell responses through enhanced MHC class I presentation, other constructs that target antigen to MHC-II presentation pathways may provide enhanced CD4+ T cell responses. This realization raises the notion of coadministration of vaccines such as E7-HSP70 and Sig/E7/LAMP-1 in a synergistic fashion. Such an approach may directly enhance both the MHC-I and MHC-II presentation of E7

and lead to significantly enhanced E7-specific CD4+ and CD8+ T cell responses and potent antitumor effects.

Although the efficacy of DNA vaccination is important, safety is also a critical issue. DNA present in the vaccine may integrate into the host genome, potentially inactivating tumor suppresser genes or activating oncogenes, thereby inducing malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation and integration should not pose any real risk [89]. A second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to pRb, a tumor suppressor protein in nuclei [78]. The presence of E7 in the nuclei may lead to accumulation of genetic aberrations and eventual malignant transformation of the host cells. To avoid such problems, strategies such as the endosomal/lysosomal-targeting Sig/E7/LAMP-1 DNA vaccine may be employed to divert E7 away from the nucleus to regions such as the lysosomal and endosomal compartments to physically separate E7 from pRb. In addition, detailed mutational analysis of E7 has led to the identification of a number of mutations that abrogate the transformation activity of E7 [38, 53, 60, 94]. Furthermore, a recent study demonstrated that a DNA vaccine encoding E7 with a mutation which inactivates Rb-binding site was able to enhance CTL activity and E7-specific antitumor effects compared to wild-type E7 [120]. Ultimately, DNA vectors employed in human clinical trials could use a minimally mutated E7 gene in which critical epitopes are preserved while potential oncogenic activity is eliminated.

Chimeric HPV VLP-Based Vaccines

Although immunization with HPV VLPs induces high-titer neutralizing antibodies in the serum and can protect animals from experimental papillomavirus infections, immunization with VLPs does not generate therapeutic effects for established or breakthrough HPV infections. Preexisting HPV infection is highly prevalent and the infected population represents an important target for the elimination of HPV-related disease, a task that likely requires the generation of protective humoral immunity as well as therapeutic T cell-mediated immunity against HPV antigens. To create a preventive and therapeutic VLP-based vaccine, several E7 chimeric VLPs consisting of the L1 major capsid protein plus the E7 protein or peptide have been created [44, 93, 111]. These E7 chimeric VLPs have been shown to generate significant E7-specific CTL activities and E7-specific antitumor effects [44, 93, 111]. Furthermore, E7 chimeric VLPs are indistinguishable from parental VLPs in their ability to elicit high titers of neutralizing antibodies [44]. These findings suggest that E7 chimeric VLPs may potentially generate L1-specific protective humoral immune responses and E7-specific therapeutic cellular immune responses in vaccinated individuals. Currently, clinical grade HPV-16 L1/L2-E2-E7 chimeric VLPs, which contain four HPV-encoded proteins (L1, L2, E2 and E7) as target antigens, are under preparation for a phase I clinical trial [Schiller, pers. commun.].

Cell-Based Vaccines

Cell-based vaccines for cancer immunotherapy can be conceptually divided into two broad categories: DC-based vaccines and cytokine-transduced tumor cell-based vaccines. DCs are the most potent professional APCs, specialized to prime helper and killer T cells in vivo. Ex vivo preparation and modification of DCs, therefore, represent an attractive vaccine strategy that is capable of enhancing T cell-mediated immunity against tumors. The understanding that DCs can be generated from hematopoietic progenitors in the setting of various cytokines, mainly GM-CSF and Flt3 ligand, has created the opportunity to use a tumor cell-based vaccine transduced with GM-CSF or Flt3-ligand cytokines to expand and prime DCs in vivo [for review, see 22].

DC-Based Vaccines

The generation of large numbers of DCs was previously hindered by a lack of information about DC maturation and the lineage-specific markers, which define their cellular differentiation state. Recent advances have revealed the origin of DCs, their antigen uptake mechanisms, and the signals that stimulate their migration and maturation into immunostimulatory APCs [for review, see 16, 52]. Several strategies for the generation of large numbers of active DCs ex vivo focus on the use of cytokine factors to induce the differentiation of primitive hematopoietic precursors into DCs [54, 57, 58, 79, 80, 150]. DCs derived from cultured hematopoietic progenitors appear to have an APC function similar to purified mature DCs. Ex vivo generation of DCs, therefore, provides a source of professional APCs for use in experimental immunotherapy. There are several vaccine strategies using DCs prepared with HPV-16 E6/E7. Vaccine strategies using DCs generated ex vivo can be classified as follows: (1) DCs pulsed with peptides/proteins and (2) DCs transduced with genes encoding HPV E6 and/or E7 through naked DNA or viral vectors.

DC Pulsed with Peptides/Proteins. Presentation of peptides derived from HPV E6 and/or E7 to the immune system by DCs is a promising method of circumventing tumor-mediated immunosuppression. Syngeneic spleen DCs pulsed with E7-specific T cell epitopes can generate protective E7-specific antitumor T cell-mediated immunity [90]. Treatment of tumors with peptide-pulsed DCs has resulted in sustained tumor regression in several different tumor models [for review, see 82]. For example, Mayordomo et al. [81] demonstrated in murine tumor models that bone marrow-derived DCs pulsed ex vivo with synthetic HPV-16 E7 peptide serve as an effective antitumor vaccine, protecting animals against an otherwise lethal tumor challenge. DCs pulsed with whole E7 protein can also generate an effective antitumor [34]. DCs pulsed with HPV-16 E7 protein are not only recognized in vitro by E7-specific CTLs but also elicit E7-specific CTL responses in vivo, which are associated with protection against a challenge with syngeneic HPV 16-induced tumor cells [34]. Another study demonstrated that DCs derived from patients can be pulsed with fusion proteins such as E6/E7 and used to generate E6/E7-specific CTLs in vitro [87].

DC Transduced with HPV E6 and/or E7 Genes. Gene-transduced DC-based vaccines represent an attractive alternative to peptide-pulsed DC-based vaccines since MHC restriction may be bypassed by directly transducing genes coding for E6 and/or E7 inside DCs, allowing synthesized peptides to be presented by any given patient's HLA molecules. Gene transfer into DCs can be accomplished by a variety of methods involving either naked DNA or the use of viral vectors. The major limitation to naked DNA transfer into DCs is poor transfection efficiency using various physical methods [1]. However, Tuting et al. [140] have described a method for the particle-mediated transfer of genes encoding HPV-16 E7 to generate DCs that express E7-MHC-I complexes. This unique method made use of plasmid DNA precipitated onto gold particles and loaded into a helium pulse gun (gene gun) to bombard bone-marrow-derived DCs evenly spread onto the bottom of a prewetted well. Not only did the vaccine successfully generate an antigen-specific CTL response in vivo, it also promoted the rejection of a subsequent, normally lethal challenge with an HPV-16-transformed tumor cell line [140]. Recently, Wang et al. [145] have successfully transduced HPV-16 E7 gene into a DC line [119] by electroporation using an HPV-16 E7 expressing vector. Wang et al. [145] demonstrated that intramuscular administration of DC-E7 generated the greatest antitumor immunity compared to subcutaneous and intravenous routes of administration. Furthermore, the study demonstrated that intramuscular administration of DC-E7 elicited the highest levels of E7-specific antibody and greatest numbers of E7-specific CD4+ T helper and CD8+ T cell precursors. These findings indicate that the potency of DC-based vaccines may depend on the specific route of administration.

Tumor Cell-Based Vaccines

The use of tumor cell-based vaccines may not be suitable for the treatment of early-stage, precancerous HPV-associated lesions because of the risks and controversy associated with administering modified tumor cells to these patients. Therefore, tumor cell-based vaccination is likely reserved for patients with advanced HPV-associated cancer. Transduction of tumor cells with genes encoding costimulatory molecules or cytokines may enhance immunogenicity leading to T cell activation and antitumor effects after vaccination [for review, see 22]. Several HPV-related tumor cell-based vaccines have been reported in preclinical model systems. For example, vaccines employing HPV-transformed tumor cells transduced with cytokine genes such as IL-12 [48] and IL-2 [13] have been demonstrated to generate strong antitumor effects in mice. Recently, an E7-expressing GM-CSF gene-transduced allogeneic tumor cell-based vaccine has been shown to generate E7-specific CTL activities and protective antitumor immunity in immunized mice [17]. These preclinical data indicate that tumor cell-based vaccines may be useful for the control of minimal residual diseases in patients with advanced HPV-associated cervical cancers.

Other Potential HPV Vaccines

HPV Nucleic Acid Vaccine Using Self-Replicating RNA Vectors

Recently, nucleic acid vaccines using RNA replicons have been shown to significantly enhance vaccine potency [153]. RNA replicon vaccines are self-replicating and self-limiting and may be administered as either RNA or DNA, which is then transcribed into RNA replicons in transfected cells or in vivo [6]. The self-replication allows expression of the antigen of interest at high levels for an extended period, optimizing vaccine potency. Since RNA-launched or DNA-launched RNA replicons eventually cause lysis of transfected cells [70, 153], the concern associated with naked DNA vaccines of integration into

the host genome is lessened. This is particularly important for vaccine development targeting E6 and E7 since HPV-16 E6 and E7 are oncogenic proteins. The RNA replicon system has recently been applied to the development of HPV vaccines. For example, several studies have demonstrated that the potency of self-replicating RNA vaccine can be further enhanced by applying the LAMP-1 targeting strategy, creating a Sig/E7/LAMP-1 RNA replicon [26, 146]. Furthermore, the potency of self-replicating RNA vaccines can be further enhanced by chimeric fusion of HPV-16 E7 to HSP70 [27, 28].

Self-replicating and self-limiting RNA replicon vaccines may be administered as DNA [6]. DNA-based RNA replicons, also known as 'suicidal' DNA, share the advantages of both RNA replicons and naked DNA vaccines without the disadvantages of either form of vaccine. Not only are they as stable and easily prepared as conventional naked DNA vaccines, they are also more potent than conventional naked DNA vaccines [6]. Since cells transfected with DNA-launched RNA replicons eventually undergo lysis (hence the term 'suicidal'), there is little concern for malignant transformation commonly associated with naked DNA vaccines. Hsu et al. [56] have recently employed DNA-launched RNA replicons for the development of HPV vaccines and have demonstrated significant E7-specific CTL activity and antitumor effects. Encouraging developments in RNA replicon vaccine development will provide the foundation for further applications in the HPV context.

Preventive and Therapeutic HPV Vaccine Using HPV Pseudovirions

Nonreplicative HPV pseudovirions containing therapeutic HPV DNA vaccines represent a new strategy for the development of preventive and therapeutic HPV vaccines. The encapsulation of naked therapeutic DNA by papillomavirus capsids has become possible using various expression systems that encode papillomavirus capsid proteins such as recombinant vaccinia viruses [157], Semliki Forest virus [100], and in vitro assembly of baculovirus-expressed capsids [139]. More recently, infectious virus particles containing a mammalian expressing DNA vector have been generated in *Saccharomyces cerevisiae* [105]. The target DNA plasmid can be packaged into HPV-16 VLPs expressed in yeast and transduced into different primary and established cells in culture and in vivo via receptor-mediated endocytosis, establishing a quantitative system to assess HPV-16 VLP infection. Such a nonreplicative papillomavirus pseudovirion provides safe and improved delivery of therapeutic DNA vaccines to target cells. Touze and Coursaget [139] have demonstrated higher frequency of gene transfer with HPV pseudovirions than with DNA alone or with liposome. These studies demonstrated that it is possible to generate papillomavirus pseudovirions in an in vitro system and that such pseudovirons can deliver packaged DNA *into* different cell lines.

Several reasons may account for the ability of pseudovirions to enhance the delivery of DNA vaccine to professional APCs. First, the capsid can protect the DNA from nuclease activity. The capsid may also act as an adjuvant. In addition, α6 integrin has been proposed as the cell surface receptor for HPV [83] and is highly expressed by the dendritic cells of the skin and lymph nodes [97]. Therefore, HPV pseudovirions may represent an ideal method to deliver therapeutic HPV DNA vaccines into DCs (either in vivo or ex vivo) to prime MHC-I-restricted CD8+ cytotoxic T cells and MHC-II-restricted CD4+ T helper cells, the most potent effector cells in antitumor immune responses. HPV pseudovirions containing therapeutic HPV DNA vaccines may, therefore, serve as ideal preventive and therapeutic vaccines.

Summary and Conclusions

In the past decade, significant progress has been made in the field of HPV vaccine development. The determination that HPV is an etiological agent for cervical cancer and their precursor lesions has paved the way for the development of preventive and therapeutic HPV vaccines that may lead to the control of HPV-associated malignancies and its potentially lethal consequences. HPV VLPs show promise as a protective vaccine capable of generating neutralizing antibodies to prevent HPV infection. Currently, a phase II clinical trial is underway, testing the HPV-16 VLP vaccine delivered intramuscularly. Neutralizing antibodies have been demonstrated in response to VLP vaccination and no significant toxicity has been observed in vaccinated patients, providing the impetus for a future phase III clinical trial. An understanding of the molecular progression of cervical cancer has led to the realization that HPV E6 and E7 are important targets for the development of HPV therapeutic vaccines for the control of established HPV infections and HPV-associated lesions. Several experimental HPV vaccine strategies including vector-based vaccines, peptide-based vaccines, protein-based vaccines, nucleic acid-

based vaccines, chimeric VLP-based vaccines, cell-based vaccines, pseudovirions, and RNA replicons have been shown to enhance virus-specific immune cell activity and antitumor responses in murine tumor systems. Several clinical trials are currently underway, based on encouraging preclinical results from these therapeutic HPV vaccines. A head-to-head comparison of these vaccines will help to identify the most potent therapeutic HPV vaccine with minimal negative side effects. Clinical HPV vaccine trials provide a unique opportunity to identify the characteristics and mechanisms of immune response that best correlate with clinical vaccine potency. Such immunological parameters will help define protective immune mechanisms for controlling HPV infections and HPV-related disease. Rational development of more effective vaccines for HPV infections would be greatly facilitated by comprehensive information on these protective immune mechanisms in humans. With continued endeavors in HPV vaccine development, we may one day control HPV infections and their associated public health problems.

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