Strategies for Developing Oral Vaccines for Human Papillomavirus (HPV) Induced Cancer using Nanoparticle mediated Delivery System

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ABSTRACT - Human Papillomaviruses (HPV) are a diverse group of small non-enveloped DNA viruses. Some HPVs are classified as low-risk as they are very rarely associated with neoplasia or cancer in the general population, and cause lenient warts. Other HPVs are considered as high-risk types because they are responsible for several important human cancers, including cervical cancer, a large proportion of other anogenital cancers, and a growing number of head and neck cancers. Transmission of HPV occurs primarily by skin-to-skin contact. The risk of contracting genital HPV infection and cervical cancer is influenced by sexual activity. Currently two prophylactic HPV vaccines, Gardasil® (Merck, USA) and Cervarix® (GlaxoSmithKline, UK), are available and recommended for mass immunization of adolescents. However, these vaccines have limitations as they are expensive and require cold chain storage and trained personnel to administer them by injection. The use of nano or micro particulate vaccines could address most of these limitations as they are stable at room temperature, inexpensive to produce and distribute to resource poor regions, and can be administered orally without the need for adjuvants in the formulation. Also it is possible to increase the efficiency of these particulate vaccines by decorating the surface of the nano or micro particulates with suitable ligands for targeted delivery. Oral vaccines, which can be delivered using particulate formulations, have the added potential to stimulate mucosa-associated lymphoid tissue located in the digestive tract and the gut-associated lymphoid tissue, both of which are important for the induction of effective mucosal response against many viruses. In addition, oral vaccines provide the opportunity to reduce production and administration costs and are very patient compliant. This review elaborately discusses different strategies that can be pursued to develop a nano or micro particulate oral vaccine for HPV induced cancers and other diseases.

INTRODUCTION

All papillomaviruses contain a double-stranded, circular DNA genome approximately 8 kb in size and normally contain eight sets of genes [1-4]. Papillomavirus types found in humans (Human papillomaviruses, HPV) are divided into five genera based on DNA sequence, and they have different life-cycle characteristics and disease associations. Among these five types, two of them, Alpha and Beta, contain about 90% of currently characterized HPVs [5-7]. The Alpha papillomavirus type is the largest group of HPVs and includes the genital/mucosal HPV types and cutaneous viruses such as HPV2. This group causes common warts and is rarely associated with cancers [8]. Whereas, Beta papillomaviruses are typically associated with non-apparent cutaneous infections in humans and also associated with the development of non-melanoma skin cancer [9, 10]. It is now clear that most HPV types, including the majority of those within the Alpha and Beta genera, cause only asymptomatic infections in immune-competent individuals and can be detected in skin swabs. Another type, Gamma also causes asymptomatic infections [11-14]. These Gamma type viruses are able to easily adapt themselves in the host, complete their life-cycle and maintain population growth, causing diseases [15, 16]. It has been found that mostly the Beta class of HPVs are involved in cancer.

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Papilloma viruses replicate and assemble exclusively in the nucleus of keratinocytes. The viruses infect keratinocytes in the basal layers of stratified epithelia and replicate in infected keratinocytes in a differentiation-dependent manner. The viral gene expression and replication proceed in a tightly controlled fashion regulated by the keratinocyte differentiation [17]. To date, more than one hundred HPV genotypes have been completely sequenced. Of which, certain types of HPVs, such HPV16, HPV18, and HPV31, are considered as high-risk or oncogenic and are frequently detected in cervical and other genital cancers. A characteristic of the infection caused by these HPVs is that viral genomes are commonly found integrated into the cancer cell genome. Other types of HPVs, such as low-risk or non-oncogenic HPV6 and HPV11, which induce benign genital warts, are very rarely found in genital malignancies [18]. The high risk HPV 16 and 18 are known to be responsible for 70% of cervical cancers worldwide, whereas HPV 6 and 11 are the predominant low-risk types that cause genital warts and recurrent respiratory papillomatosis (RRP) [19]. HPV-caused human cervical cancer results in the second largest of cancer related deaths of women in the world. In the United States, it became the center of attention when a study showed that 25% of persons between the ages of 14 and 19 and 45% of persons between the ages of 20 and 24 were HPV positive. It is estimated that more than 80% of both men and women in the United States will be infected with HPV at some point in their lives [20, 21]. Estimated yearly cervical cancer cases and deaths associated with HPV are 490,000 and 270,000, respectively [20, 21]. Sexually transmitted HPV is a necessary factor for the development of cervical cancer and its precursor lesions. Cervical HPV infection is found in 5–40 percent of asymptomatic women of reproductive age [22]. Risk of infection increases with increased number of sexual partners, starting sexual intercourse at a younger age, and recent acquisition of new partners [23]. Although the vast majority of these infections are transient, a substantial increase in risk of cervical neoplasia exists for women who develop persistent, long-term infections with oncogenic HPV types [23-26]. Currently, two prophylactic HPV vaccines, Gardasil (Merck, USA) and Cervarix (GlaxoSmithKline, UK) are available and recommended for mass immunization of preteen girls and boys at age 11 to 12 years. Recently the CDC also recommended the HPV vaccines for teen boys and girls who did not get the vaccine when they were younger; including teen girls and young women through age 26, and teen boys and young men through age 21.

Both Gardasil and Cervarix vaccines consist of the immunogenic proteins L1, which are the major proteins of the capsid of papillomavirus. L1 proteins self-assemble into 'virus-like particles' (VLPs) when expressed at high levels in cultured cells. VLPs are multi-protein structures that mimic the organization and conformation of authentic naïve viruses but do not contain any genetic material. When administered, VLPs are able to generate immunity as if the immune system has been confronted with a real virus [27]. However, because VLPs do not contain any genetic material, they are unable to replicate and as such are harmless and safe. Cervarix vaccine contains VLPs of HPV types 16 and 18, whereas Gardasil includes additional VLPs of HPV types 6 and 11 [28]. The VLPs used in Gardasil are manufactured from *Saccharomyces cerevisiae* (bread yeast) which is transfected with the genes expressing L1 whereas Cervarix VLPs are manufactured from *Trichoplusiani* insect cell line that was infected with L1 encoding recombinant baculovirus. Gardasil and Cervarix have 3 years and 4 years shelf life respectively, and are stored at 2 to 8º C. The approximate cost for three doses is about $400 to $500 [29]. The adjuvant in Gardasil is amorphous aluminum hydroxyphosphate sulfate (AAHS) (225 µg), whereas aluminum hydroxide (500 µg) and 3-O-deacyl-4' monophosphoryl lipid A (50 µg) are the adjuvants in Cervarix [30]. Both vaccines are administered intramuscularly in a volume of 0.5 mL and require multiple doses. After the administration of the prime dose, two more booster doses are administered, one within 1 to 2 months and another within 6 months. While both Gardasil and Cervarix are shining examples of bench to bedside research, these vaccines have significant drawbacks that limit their applications in the settings where they are most needed. They are expensive, and require cold chain storage and trained personnel to administer them by injection. In addition, there is a growing concern regarding their adverse effects. Patients receiving Gardasil and Cervarix may experience pain, fatigue, redness, swelling, fever, GI symptoms (diarrhea, nausea, vomiting), headache, dizziness, myalgia and arthralgia [31-35]. The most common adverse

effect is injection-related local reaction, such as pain, swelling and erythema with a rate of 95% of light to moderate intensity [32, 33]. Severe adverse effect, such as severe headache with hypertension, gastroenteritis and bronchospasm, were also noted [32]. There are more data available on adverse effect associated with Gardasil than Cervarix; however, the major adverse effect for the latter vaccine is also injection-related local pain (78%) [32]. Other disorders that Gardsil may cause include infection and infestation (52%), gastrointestinal disorder (13.4%), nervous system disorder (9.4%), and reproductive and breast disorders (24.8%). Darja Kanduc has shown that HPV 16 antigen can induce autoimmune reaction against human proteins which might lead to pathologies such as spinal muscular atrophy, proximal muscle weakness that cause maddling gait, toe-weakening, lordosis, frequent falls, difficulty in standing up and climbing stairs, cardiovascular and musculskeletal abnormalities, disorder of lipoprotein metabolism leading to hypercholesterolemia, and increased proneness to coronary artery disease [36]. The aluminium adjuvant in both vaccines has also been shown to cause adverse effects. Stephanie Seneff has shown that children may not react acutely to the aluminum adjuvated vaccine, which can lead to neural damage that is partly mediated by exuberant production of nitric oxide [37].

Thus it is very important to develop an affordable, safe and highly effective HPV vaccine to fight virus induced diseases globally. To develop a highly efficient and cost effective vaccine, several factors must be taken into consideration. The vaccine formulation must be safe and easy to administer. The vaccine should address the issue of adverse effect. The vaccine formulation should also be capable of eliciting the desired immune responses, humoral and/or cellular mediated. An ideal vaccine should require least number of doses without the need for a booster dose(s). It is also important that all components of the vaccine are commercially available, safe, affordable and nontoxic. The process of vaccine manufacturing should be easy, affordable, and amenable to other steps of preparation such as sterilization, lyophilization, spray drying or vacuum drying, packaging and reconstitution of the dried powder. The vaccine formulation should be stable with respect to size, surface morphology and size distribution throughout the process of preparation, storage and administration. Moreover, the antigen has to be chemically and physically stable throughout the process of antigen loading and there should be no premature release/leakage of antigen. To address the issues with the currently available HPV vaccines and to prepare a highly efficient and cost effective alternative vaccine based on the aforementioned criteria, a particulate formulation of nano or micron size is considered to be the most desirable candidate. Nano or micro particulate formulations of vaccines have the potential to overcome the limitations of currently available vaccines as the nano or micro particulate formulations may be stable at room temperature and can be administered orally. In this review, we discuss the different aspects of developing a highly efficient, stable and cost effective HPV vaccine using nano/micro particle for oral administration.

VLP-BASED HPV VACCINES

The first vaccination against HPV was demonstrated by Shope in 1937 where neutralizing antibodies protected rabbits against high-dose viral challenge with cottontail rabbits papilloma virus (CRPV). The study found that generating serum neutralizing antibody to the virus capsid protein is an effective strategy for prophylactic vaccination against the infection [38]. The currently available HPV vaccines, Gardasil and Cervarix, are subunit vaccines consisting of VLPs assembled from the major L1 proteins of HPV type 16, 18, 6 and 11 (Gardasil) and HPV type 16 and 18 (Cervarix). As the VLPs have no genetic material in them and cannot grow or cause any infection, there are huge opportunities for using VLPs as antigens in virus-causing diseases that are hard to cure. There are numerous advantages of using VLPs as antigens in vaccine formulation. VLPs are excellent prophylactics because they are self-assembling bio-nanoparticles (20 to 60 nm in diameter) that expose multiple epitopes on their surface and very accurately mimic the native virions [39]. In addition, VLPs are superior to bacterial vaccines and viral antigens in a number of ways and bacterial antigens can sometimes revert to the virulent form [40, 41]. On the other hand, in the case of viral antigens, the authentic and attenuated virions cannot be used as antigens in a prophylactic vaccine because they would contain oncogenic viral genomes that would be infectious [42]. A VLP has no such side effects and can eliminate these risks. VLPs not only resemble authentic

virions morphologically, but they also mimic virions immunologically, which means that they are able to induce high titers of neutralizing antibodies to conformational epitopes when vaccinated [43, 44]. The surface of VLPs consists of an array of antigenic epitopes that mimic the surface of native virions more reliably than specific isolated subunits or subcomponents of the virus [43]. VLPs can be produced in either a prokaryotic or eukaryotic cells by expressing the protein in a different medium such as mammalian cells, insect cells, yeast, or even bacteria [45].

Both Gardasil and Cervarix vaccines induce the generation of high concentrations of neutralizing antibodies to L1 and have been shown to be highly efficacious in randomized and controlled trials. Instead of flagging the antigen, the antibodies are able to neutralize the biological effect of the antigen. It has also been shown that the neutralizing antibodies cause cell-mediated cytotoxicity to the virus [46]. Mechanistic studies of the HPV infection revealed that the virus first causes microabrasion and removal of the full thickness of the epithelium but keeps the epithelium basement membrane intact since the virion attaches first to the basement membrane before entering basal cells. In the epithelial basement membrane, the virus binds to the heparin sulfate proteoglycans via L1 protein. The virus capsid then undergoes a conformational change and allows the exposure of L2 protein that binds to the surface molecules of keratinocytes. The capsid then undergoes further conformational changes, leading to the exposure of cellular receptor binding sites on L1 protein. Subsequently, the virus binds to cellular receptor via L1 protein and enters the cell [47]. Following HPV L1 VLP immunization, antibodies are produced which prevent both initial binding of HPV virus to the basement membrane and binding of the virus to the keratinocyte cell surface [48]. It has been shown that the antibodies to L1 are effective at very low concentrations, consistent with data from the animal papilloma virus model and from natural infections in humans [49, 50]. The virus-like-particle can be produced by expressing the specific HPV protein in eukaryotic cells. VLPs in Gardasil are produced in yeast cells by cell disruption and purified by a series of chemical and physical methods. Cervarix VLP, on the other hand, is produced in insect cells. Asghar et al have also reported the production of recombinant HPV-16L1 protein in Eukaryotic Sf9 insect cells. The recombinant protein L1 was predominantly ~ 60 kD indentified by western blot analysis. VLP formation was confirmed by SDS-PAGE with distinct immunoreactivity in western blot analysis and electron microscopy [51].

The HPV infection causes several manifestations, including common warts, epidermodysplasia verruciformis, anogenital warts, cervical and vulvar cancer of the penis, vagina and anus, and recurrent respiratory papillomatosis [52]. Although HPV is asymptomatic and auto-limited, it is a public health concern because of its association with genital tract malignant disease among men and women [53]. HPV genital infection is mainly transmitted by genital-to-genital contact often during sexual intercourse. Both of the currently available HPV vaccines are VLP-based vaccines. They are classified as the quadrivalent HPV vaccine (Gardasil) and the oncogenic HPV bivalent vaccine (Cervarix) [54]. Clinical studies have shown that the quadrivalent vaccine offers protection against persistent HPV infection; cervical, vaginal and vulvar lesions that are precursors for cancer; and genital warts caused by HPV types 6, 11, 16 or 18 in women aged 16 to 26 years old who were not previously infected by these HPV types [54]. On the other hand, the bivalent vaccine contains only VLPs of oncogenic HPV types 16 and 18. Cancer vaccines are medicines that belong to a class of substances known as biological response modifiers. Biological response modifiers work by stimulating or restoring the immune system's ability to fight infections and disease. There are two broad types of cancer vaccines: preventive (or prophylactic) vaccines, which are intended to prevent cancer from developing in healthy people; and treatment (or therapeutic) vaccines, which are intended to treat an existing cancer by strengthening the body's natural defenses against the cancer. Cancer preventive vaccines target infectious agents that cause or contribute to the development of cancer. They are similar to traditional vaccines, which help prevent infectious diseases, such as measles or polio, by protecting the body against infection. Both cancer preventive vaccines and therapeutic vaccines are based on antigens that are carried by infectious agents and that are relatively easy for the immune system to recognize as foreign. The current HPV vaccines, Cervarix and Gardasil, are prophylactic vaccines designed to reduce the occurrence of cervical

cancer. Although both vaccines have been proven to be highly effective, the limitation of these vaccines is their cost; they are expensive in terms of preparation and preservation. In addition, the administration of these vaccine requires trained personnel. Due to these limitations, mass application of these vaccines around the world is severely hindered. The ultimate goal of vaccination is to ensure the production of strong and lasting immune responses after a single dose of antigen without the need for booster doses [55, 56]. In order to ensure the quality and quantity of the immune response, it is highly important that the immune system is presented with antigens at the right location of the targeted pathogen in sufficient amount [57, 58]. Nano or micro particulate formulations of vaccines have the potential to address most, if not all, of these limitations as they may be stable at room temperature, inexpensive to produce, more effective as a particulate carrier, and can be administered orally. A biodegradable polymer based particulate vaccine can act as an adjuvant itself, therefore there is no need for using salt based adjuvants, which will eliminate the adverse effects caused by adjuvants.

In addition, it is also possible to increase the efficiency of the particulate vaccine by adding appropriate ligands, charged particle or any other biocompatible chemical to increase the specificity of the nano or micro particles for targeted delivery [59]. Human papilloma virus (HPV)-16 is the prevalent genotype associated with cervical tumours. Virus-like-particle-based vaccines have proven to be effective in limiting new infections of high-risk HPVs, but the high cost has hampered their use, especially in poor developing countries. Avipox-based recombinants are replication-restricted to avian species and represent efficient and safe vectors for immunocompromised hosts. These recombinants can elicit a complete immune response. A new fowlpox virus recombinant encoding HPV-L1 (FPL1) was engineered and evaluated side-by-side with a FP recombinant co-expressing L1 and green fluorescent protein (FPL1GFP). This fowlpox virus recombinant correctly express the L1 in vitro in different cell lines which was confirmed by western blot, immunofluorescence, real-time PCR, and electron microscopy. Mice were also immunized to determine its immunogenicity. It was also demonstrated that the FPL1 recombinant better expresses L1 in the absence of GFP, correctly assembles structured capsomers into virus-like particles (VLPs), and elicits an immune response in a preclinical animal model. Thus far this is the first report of HPV VLPs assembled in eukaryotic cells using an avipox recombinant [59].

OTHER HPV VACCINES

Particulate vaccine can deliver a wide variety of antigens such as attenuated, killed or inactivated pathogens, recombinant protein, peptides from oncogenic protein, synthetic peptide, carbohydrates, lipids and DNA. Biodegradable polymer based nanoparticle can be used as a suitable carrier for the development of effective and affordable DNA and protein subunit vaccines. Rational development of such vaccine formulations requires a detailed understanding of their physico-chemical properties, cell-free environment and in vitro behavior. Also it is necessary to understand the process of particle uptake and processing mechanisms of antigen presenting cells (APC), which are capable of stimulating safe and effective immune responses.

One effective vaccine is peptide vaccine, which offers several advantages over classical vaccines. However, peptides alone are not immunogenic and need a delivery system that can boost their recognition by the immune system. In recent years, nanotechnology-based approaches have become one of the most promising strategies in peptide vaccine delivery [60]. In case of HPV vaccine, peptides can be obtained from the Human Papillomavirus (HPV) E6 and E7 oncogenes and can be an effective antigen to develop a therapeutic vaccine for HPV induced cancers. These peptide sequences derived from the oncogenic E6 and E7 viral proteins have been shown to represent suitable tumor associated antigens (TAAs) for cervical cancer and are considered as ideal candidates for developing therapeutic vaccines [61-63]. These peptides are easily recognized by CD8 T lymphocytes, which are the most effective components of the adaptive immune system capable of recognizing and destroying viral-infected and transformed malignant cells [64-66]. In addition to peptides obtained from the E6 and E7 viral proteins, synthetic peptides representing these TAAs have also been tested in numerous ways in human patients and mouse cancer models for their ability to generate anti-tumor T cell responses capable of exhibiting anti-tumor effects [67-69]. However, a significant limitation observed of these

antigens is that they show only a modest T cell responses capable of dealing with very early disease stages. Therefore it is necessary to develop improved peptide-based immunization strategies which will have significant impact against advanced disease stages. Kelly Barrios et al has developed a synthetic peptide vaccination strategy, called TriVax, that is effective in generating vast numbers of antigen-specific T cells in mice capable of persisting for long period of time [70]. They have described an improved peptide vaccination strategy in mice that shows a significant immune response involving a large number of CD8 T cells [71, 72]. The vaccine TriVax , using HPV16-E749–57, induced large and persistent T cell responses that were therapeutically effective against established HPV16-E7 expressing tumors. In most cases, TriVax was successful in acting against 6–11 day old tumors. In addition, TriVax induced long-term immunological memory, which prevented tumor recurrences. The TriVax vaccine consists of a synthetic peptide corresponding to the minimal T cell epitope, poly-IC adjuvant and costimulatory monoclonal anti-CD40 antibodies (αCD40 mAb), which are mixed together and administered intravenously.

More recently, Rahimian has attempted to develop a HPV cancer vaccine formulation [73]. Synthetic long peptides (SLPs) derived from HPV16 E6 and E7 oncoproteins have been used for therapeutic vaccination. In preclinical and clinical studies, adjuvants based on mineral oils (such as incomplete Freund's adjuvant (IFA) and Montanide) are used to create a sustained release depot at the injection site. While the depot effect of mineral oils is important for induction of robust immune responses, their administration is associated with severe and long lasting adverse effects. In order to develop an alternative to mineral oil based vaccine, polymeric nanoparticles (NPs) based on hydrophilic polyester (poly(d,l lactic-co-hydroxymethyl glycolic acid) (pLHMGA)) were prepared. These NPs were loaded with a synthetic long peptide (SLP) derived from HPV16 E7 oncoprotein and a Toll like receptor 3 (TLR3) ligand (poly IC) by double emulsion solvent evaporation technique. The therapeutic efficacy of the nanoparticulate formulations, was compared to that of HPV SLP+poly IC formulated in incomplete Freund's adjuvant (IFA). The results showed that the encapsulation of HPV SLP antigen in NPs substantially enhanced the population of HPV-specific CD8+ T cells when combined with poly IC either co-encapsulated with the antigen or in its soluble form. Although the therapeutic efficacy of NPs containing poly IC in tumor eradication was equivalent to that of the IFA formulation, the administration of pLHMGA nanoparticles was not associated with adverse effects and therefore these biodegradable nanoparticles are excellent substitutes for IFA in cancer vaccines.

A DNA vaccine can be another alternative to fight the HPV infection. DNA vaccines have emerged as an attractive approach for antigen-specific T cell-mediated immunotherapy to combat cancers. In HPV infection, two oncogenic proteins, E6 and E7, are consistently co-expressed in HPV-expressing cervical cancers and are important in the induction and maintenance of cellular transformation. Therefore, immunotherapy targeting E6 and/or E7 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. Chien et al has shown that a DNA vaccine can be effectively used against HPV infection [74]. In the case of HPV, T cell-mediated immunity is one of the most crucial components in our defense against HPV infections and HPV-associated lesions. Therefore, the goal of DNA-based vaccination is to generate strong E6/E7-specific T cell-mediated immune responses. Intradermal administration of DNA vaccines via a gene gun represents an efficient way to deliver DNA vaccines into professional antigen-presenting cells *in vivo*. Professional antigen-presenting cells, such as dendritic cells, are the most effective cells for priming antigen-specific T cells. Using the gene gun delivery system, several DNA vaccines that employ intracellular targeting strategies for enhancing MHC class I and class II presentation of encoded model antigen HPV-16 E7 were tested. Furthermore, a strategy to prolong the life of DCs to enhance DNA vaccine potency was developed. More recently, a strategy to generate antigen-specific CD4+ T cell immune responses to further enhance DNA vaccine potency was also developed. The impressive preclinical data generated from ourthese studies have led to several HPV DNA vaccine clinical trials.

A live bacterial-based HPV vaccine can be another choice to fight HPV infections. Bacterial-based vaccines are inexpensive and feasible to prepare in a regular laboratory set up. Yan et al evaluated the potential value of live attenuated

Shigella. flexneri 2a sc602 strain-based HPV16L1 as a high-efficiency, low-cost HPV16L1 mucosal vaccine [75]. The study revealed that the recombinant sc602/L1 vaccine induced high L1-specific systemic and mucosal immune responses as well as cell-mediated Th1 and Th2 immune responses in guinea pig model. Sc602/L1 vaccine induced higher L1-specific IgG and IgA antibodies as well as HPV16-neutralizing antibodies in genital region in sc602/L1 mucosal immunized animals than in L1 intramuscular immunized animals. Though both are via mucosal delivery, immunized sc602/L1 vaccine by rectum route induced higher L1-specific IgA and IgG titers in genital region than by conjunctiva route. In addition, sc602/L1 also strongly increased L1-specific IFN-γ and IL-4 expression, implying its effect on cell-mediated immune response. The study proves that sc602/L1 bacterial-based vaccine may have a significant protective effect against HPV infection.

NANO-PARTICULATE VACCINES

Emerging nanotechnology in medical science has provided an unparalleled opportunity to advance the treatment of various severe diseases. Nanoparticles exhibits several unique properties, such as higher surface-to-volume ratio, small size, ability to encapsulate various drugs, and tunable surface chemistry, which give them many advantages over their bulk counterpart. These advantages include multivalent surface modification with targeting ligands, efficient navigation of the complex in an in vivo environment, increased intracellular trafficking, addition of any charged particles to increase target selectivity and sustained release of drug. These advantages make nanoparticles an ideal candidate for formulating drugs for most prevalent and challenging diseases including cancer [76]. Nanoparticulate drug carriers are passively targeted to cancer tumors using the enhanced permeability and retention effect (EPR) in tumor area, thus they are the most suitable contestant for the delivery of chemotherapeutics in cancer treatment. In fact, advances in nanomedicine have rapidly made possible some of these drugs to be used in clinical practice. To date, there are five nanoparticle chemotherapeutics that have been approved for cancer treatment and many more are under clinical investigation [77].

In addition, to their therapeutic use, nanoparticles can also be useful as a new strategy for vaccine development. Current successful vaccines are live, attenuated, killed or fragmented pathogens. However, due to their complex nature, such vaccines can vary in quality from batch to batch and can induce adverse effects such as those associated with the whole pertussis, Sabin polio, measles, respiratory syncytial virus (RSV) or rotavirus vaccines [78-81]. A particulate formulation has huge potential in vaccine development as the particle can be used as antigen career and/or adjuvant and can address the issue of adverse effects that are caused by conventional vaccines. New vaccine strategies can take advantage of particulate compounds – especially nanoparticles – to target antigen presenting cells more efficiently [82]. Particulate formulations offer a number of advantages in vaccine development. Particulate carriers can serve as an effective antigen delivery system that is able to enhance and/or facilitate the uptake of antigens by antigen-presenting cells (APCs) such as dendritic cells (DCs) or macrophages [83, 84]. Particle-based antigen carriers can also serve as a depot for controlled release of antigen, thereby increasing the availability of antigen to the immune cells. It has been found that antigen release may enhance not only the level of the immune response but also its quality [85, 86]. In addition, particle-based adjuvants possess the ability to modulate the type of induced immune responses when used alone or in combination with other immune-stimulatory compounds [87]. Particulates have the ability to protect the integrity of antigens against degradation until delivered to the immune cells [88]. This is particularly important in oral vaccine formulations where antigens must be protected from the harsh acidic conditions of the stomach and enzymatic degradation in the GI tract [89]. More importantly, particulate vaccines can potentially cross-present the antigen; antigen cross-presentation is especially important to generate CD8+ T-cell responses against viral infections [90, 91]. Another advantage of using particulate formulation of a vaccine is that it can eliminate the use of adjuvants which do not have much immunogenic effect. For example, the vaccine Cervarix contains both aluminium hydroxide and ASO4 (3-O-desacyl-4-monophosphoryl lipid A) as adjuvants. Theses adjuvants only improve humoral immunity but do not contribute to cell mediated immune response, the main immune function of the VLP [92, 93]. The immunologic effect of particulate vaccines is

related to the size, stability, antigen loading and antigen-release kinetic properties of the particle [94]. The immune response is also influenced by particle interaction with APCs and antigen presentation and processing by APCs [95]. Several synthetic polymers are used in the preparation of the particles such as poly (lactic acid) (PLA), poly(ortho esters), poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol, and polyphosphazene. Natural polymers such as albumin, gelatin, collagen, chitosan and alginate have also been used in vaccine candidate formulations [96-99]. When compared to micro particles, nano sized particles offer more options as the surface ratio is higher when the size is downgraded to the nano scale [100]. Advantages of nanoparticle-based delivery of vaccines/drugs, include improved biological stability of antigen/drug and efficacy in targeting APCs for induction of innate and adaptive immunity due to Class I and Class II presentations [101]. Nanoparticles may also provide enhanced intracellular concentrations, controlled release of vaccine antigen/drug, and reduced number of administrations due to enhanced immune response.

One of the most significant advantages of nano size particle is that the particle can act as immune-stimulating adjuvant. Gamvrellis, et al. have shown that a nano-particulate antigen delivery system was able to induce a substantial immune response without inducing any inflammation. The antigen appears to induce substantial immune responses in mice and sheep without adding stimulators such as toll-like receptors or other pathogen recognition receptors [102]. Nano particulate formulations can also be used to develop a safe and effective cancer vaccine formulations. Poly (d, l-lactic-co-glycolic acid) nanoparticles (PLGA-NPs) can be used to formulate a cancer vaccine delivery system which has potential in the development of future therapeutic cancer vaccines [103]. This nano particle can target dendritic cells (DCs) which can effectively initiate antitumor activity. The PLGA nano particle containing antigens along with immune-stimulatory molecules (adjuvants) can not only target antigen actively to DCs, but also provide immune activation and rescue impaired DCs from tumor-induced immuo-supression [104]. The authors further assessed the extent of maturation of DCs after treatment with the antigen, monophosphoryl lipid A (MPLA), and encapsulated PLGA nanoparticles. The generation of primary T-cell immune responses elicited by DCs was monitored. Results showed that the high amounts of pro-inflammatory and TH1 (T helper 1) polarizing cytokines and chemokines released by the nanoparticles are greater than that achieved by MPLA in solution [105]. These results confirmed that the nanoparticle formulation of a vaccine is more immunogenic when compared to the solution form of the antigen. Dendritic cells in peripheral tissues are important as they act as sentinels of the immune system, detect and capture pathogens entering the body, and present their antigens to T cells to trigger responses directed towards the elimination of the pathogen. Diwan et.al. investigated the formulation of a pharmaceutically acceptable, biodegradable, and strategic nanoparticulate delivery system and its application for efficient antigen loading of DCs to achieve antigen specific T cell activation. The results of the investigation indicated that PLGA nanoparticles are able to mimic certain features of pathogens and can efficiently act as delivery systems for targeting vaccine antigens to DCs and activating potent T cell responses [105].

In the intestine, particles are readily phagocytized by the antigen presenting cells, mostly microfold cells (M cells) which are present in the underlying region of the Peyer's patches of the small intestine [106-108]. The first step of mucosal immune response is the trans-epithelial transport of antigens and pathogens. The antigen then reaches the site of immune response. The delivery of antigens across the epithelial barrier to the underlying lymphoid tissue is mediated by M cells, a specialized epithelial cell type that occurs only in lymphoid follicle associated epithelium. Particulate formulation of vaccines where antigens are coupled to or encapsulated are found to be transported through the epithelial layer by M cells more efficiently than live, attenuated, killed pathogens or antigens. In case of particulate vaccine, it is also possible to enhance the binding capacity, target ability and uptake of the particle by adding ligand, charges particle at the surface. Such modification leads the particulate vaccine to the receptors on the M cell surface [109]. These M cells then transport the particles to the macrophages or other cells underlying the gut-associated lymphoid tissues [110, 111]. Thus, M-cell targeting lectins, such as Ulex Europaeus Agglutinin (UEA-1), Aleuria Aurantia Lectin (AAL), and Wheat Germ Agglutinin (WGA), can

increase cellular uptake and efficiency of particulate vaccine and can be added to the formulation to enhance targeting of the Peyer's patches. Further immune-stimulatory cytokines such as IL-1 and IL-12 can be added for enhanced immunity. Since the antigen is presented in a particulate formulation, there is no need for added adjuvants due to the sustained nature of antigen release from the particles. Several technologies for the oral administration of drugs/vaccines using nanoparticles, microparticles (microspheres), and a number of biodegradable polymer-based microparticle formulations have been studied as effective delivery systems [108, 112]. Biodegradable and biocompatible polymers, copolymers and lipids have been used to prepare nano/micro-particle as vaccine-delivery systems [113-115]. The material is selected based on several factors, including biocompatibility, degradation rate, hydrophilicity or lipophilicity, surface charge, and polarity. Examples of nanoparticle-based vaccines include oral biodegradable microspheres with recombinant anthrax vaccine for immunization against anthrax infection, poly (DL-lactide-co-glycolide (DL-PLG) microspheres encapsulating phosphorylcholine against *Salmonella typhimurium*, and albumin-chitosan mixed matrix microsphere-filled coated capsule formulation of the typhoid vaccine [114-116].

POTENTIAL BENEFITS OF AN ORAL NANO/MICRO-PARTICULATE HPV VACCINE

Oral administration is the most preferred route for drug delivery as it is most patient compliant. Therefore developing an oral HPV vaccine with high efficiency and low cost will eliminate the limitations that the current vaccines have. The oral route will also eliminate the need for a trained personnel to administer the vaccine, which will give the vaccine a more global character as it will be easily available and applicable in resource poor countries. In addition, oral vaccines have the potential to stimulate mucosa-associated lymphoid tissue (MALT) located in the digestive tract and the gut-associated lymphoid tissue (GALT). Both of these tissues are important for the induction of an effective mucosal response against many viruses [117]. For Human papillomavirus, an elevated mucosal immune response could serve as a first line of protection against the infection. Alternative immunization routes for HPV other than the intramuscular route of administration have been investigated. The oral delivery of vaccines as an alternative immunization route and the efficiency of mucosal immunization for different antigens have been described [118]. In addition the intranasal route of administration for vaccine delivery has been investigated. Results from studies of both oral and intranasal routes of administration show the potential of mucosal immunization with HPV VLPs for inducing a neutralizing antibody response and L1-specific cytotoxic T-lymphocytes [118, 119].

Several animal studies were conducted using HPV L1 VLPs or different assembly forms (capsomeres) in the form of a solution, or in edible products (HPV L1 VLP expressed in potato) [120]. Recombinant clones of attenuated *Salmonella enterica* (Serovar Typhi and Typhimurium) strains expressing HPV-16 and HPV-18 L1 antigens were also shown to induce a strong immune response and are currently in the pre-clinical testing phase for oral or mucosal administration [121]. However, oral vaccine formulations without adjuvants have thus far required large amounts of antigens compared to the intramuscular route when delivered in solution form. Nano or micron sized particles may provide enhanced intracellular concentrations, controlled release of antigens, stability, and a reduced number of administrations. It has been shown that microparticles prepared from biodegradable polymer can be easily prepared, well characterized, administered orally and be a reliable career of variety of drugs and vaccine antigen such as oligonucleotide antisense to NF-kB, plasmid DNA encoding hepatitis-B surface antigen [122, 123]. Studies have shown that microparticulated formulation increases the bioavailability of orally administered antisense. Antisense drug is considered as next generation drug due to their specific targeting ability to mRNA and minimum toxic effect. However, the drug has poor biological stability, short half-life and limited cellular uptake [124]. The bioavailability of antisense solution via oral administration was only 9%, whereas the bioavailability of the antisense encapsulated in bovine serum albumin increased up to 70% [125]. The adjuvant-like properties of the nanoparticles also enhance the immune response due to their ability to target Peyer's Patches and M cells (microfold cells). Peyer's Patches are aggregations of lymphoid tissue normally found in

the lowest portion of the small intestine and are full of M cells. To evoke the mucosal immune response, antigens on the mucosal surface must first cross the impermeable epithelial barrier into lymphoid structures such as Peyer's Patches where the M cells take up the antigen and then process it and present it to the antigen cells such as macrophages and dendritic cells. This process, called antigen transcytosis, is mediated mainly by M cells [126]. A potential problem with a vaccine's efficacy is the lack of the vaccine's targeting ability. Thus, addition of any targeting ligand to the particle or modification of the particles in a way that they can target M cells in Peyer's Patches enhances the vaccine's efficiency. One such modification can be done with Chitosan. Chitosan is a positively charged polysaccharide that can be used to provide a positive charge to the surface of the vaccine particle which will then enhances its ability to target M cells, as the surface of M cells is negatively charged. Also a ligand such as AAL (aurantia aleuria lectin), which is very specific to some receptors at the surface of M cell layers, can increase the targeting ability of the vaccine. In addition, cytokines such as IL-2 and IL-12 are able to enhance the immune response. The combination of all of these strategies can produce a more efficient vaccine as well as circumvent the issues with the current intramuscularly administered VLP solution vaccines.

CONCLUSION

HPV-caused cancers and diseases remain an important health concern in the United States and throughout the world. Thus far there are only two vaccines available for HPV related cancers and other diseases. These vaccines are proved to be highly efficient in terms of preventing diseases, however, they have several disadvantages which limit their use, particularly in resource-poor countries. Furthermore, the side effects of these intramuscular vaccines are raising important health questions. Therefore, there is a great need for developing a new alternative HPV vaccine which will be cost effective and can significantly contribute to global public health. There are a number of choices as alternatives to the current VLP- based HPV vaccines, such as a DNA based vaccine, peptides from HPV oncogenic protein, synthetic peptides, and live bacteria. The problem with these vaccines alternatives is that their delivery system is inefficient. A biodegradable polymer based nanoparticle is the most suitable formulation to address this delivery issue. Evidence suggests that it is possible to develop an alternative oral HPV vaccine using a nano or micro particulate formulation that promises to be highly efficient, more cost effective, and more patient compliant than the existing formulations. Studies have shown that VLPs, which are multi-protein structures that mimic the exact organization and conformation of native viruses but lack the viral genome, are perfect for preparing safer and cheaper vaccine candidates. In addition, studies have also revealed that smaller size VLPs such as nano or micron size offer numerous advantages in vaccine development when compared to solution form. Thus, combining VLP technology with biodegradable polymer based nano particulate formulations appears to be a very promising new approach for the future development of desirable oral HPV vaccines.

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