

Development of a Topical Protein Therapeutic for Human Papillomavirus and Associated Cancers

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Contents

Abstract	209
1. Human Papillomaviruses (HPVs) and Human Disease	209
1.1 Papillomavirus Biology	210
1.2 Cell Transformation by HPV	211
1.3 Prophylactic Vaccines	212
1.4 Therapeutic Vaccines	212
1.5 New Approaches to Treatment	212
2. The E2 Protein	212
2.1 The Host Immune Response to E2	213
2.2 E2 and Cell Proliferation	213
3. The Delivery of E2 to HPV-Transformed and HPV-Infected Cells	214
3.1 Viral Delivery of E2	214
3.2 Non-Viral Delivery of E2	214
3.3 Targeting E2 to Tumor Cells and Other Challenges	215
4. Conclusion	215

Abstract

Human papillomaviruses (HPVs) are the causative agents of several disease states, including genital warts and cervical cancer. There are around 500 million cases of genital warts per annum worldwide and around 450 000 cases of cervical cancer. Although HPV vaccines should eventually reduce the incidence of these diseases, new and effective treatments are still urgently required. The E2 (early) proteins from some HPV types induce growth arrest and apoptosis, and these proteins could be used as therapeutics for HPV-induced disease. A major obstacle to this approach concerns the delivery of the protein to HPV-transformed cells and/or HPV-infected cells *in vivo*. One possible solution is to use recombinant viruses to deliver E2. Another possible solution is to use purified E2 proteins or E2 fusion proteins. The herpes simplex virus VP22 protein is one of a small number of proteins that have been shown to cross the cell membrane with high efficiency. VP22-E2 fusion proteins produced in bacterial cells are able to enter mammalian cells and induce apoptosis. This suggests that VP22-E2 fusion proteins could be topically applied as a treatment for HPV-induced diseases, most probably post-surgery. In this review, we discuss this and other approaches to the topical delivery of selective therapeutic agents against HPV-associated conditions.

1. Human Papillomaviruses (HPVs) and Human Disease

Papillomaviruses are associated with a variety of benign and malignant lesions. Over 100 human papillomaviruses (HPVs) have been classified on the basis of DNA sequence homology, and

at least 40 of these infect the genital tract (reviewed by de Villiers^[1]). These viruses can be divided into two groups. The so-called 'high oncogenic risk' (HR)-HPV types, such as HR-HPV 16 and HR-HPV 18, are causative agents of several human tumors (reviewed by zur Hausen^[2]). In contrast, the 'low oncogenic risk'

(LR)-HPV types, such as LR-HPV 6 and LR-HPV 11, cause benign genital warts.^[3]

Benign genital warts (condylomata acuminata) induced by the LR-HPV types are the most common sexually transmitted disease. There are >5 million new cases of sexually transmitted LR-HPV infections in the US each year, and at least 20 million North Americans are infected at any given time.^[4] The current orthodox treatments for genital warts include surgery, laser therapy, cryotherapy, electrocautery, nonselective cytotoxic compounds (such as podophyllin), and imiquimod, an agent thought to stimulate the immune response to infection.^[5] These treatments are invasive, and in many cases ineffective. Many unorthodox treatments of dubious value are also available, perhaps indicating the level of ineffectiveness of the orthodox treatments. LR-HPV types can also cause recurrent laryngeal papillomas in young children, and frequent surgery can be required to maintain an open airway.^[6] Novel approaches to the treatment of these HPV-associated benign lesions would clearly be very useful.

HR-HPV types are a causative agent of cervical cancer.^[7] Cervical cancer is at the end of a continuum of disease that extends from cervical intraepithelial neoplasia (CIN) 1 (mild dysplasia), CIN 2 (moderate dysplasia), and CIN 3 (severe dysplasia and carcinoma *in situ*) to cervical carcinoma. HR-HPV infection is thought to initially bring about the formation of CIN 2. In a minority of cases, these lesions progress to CIN 3 and eventually to invasive carcinoma. HR-HPV infection alone is generally thought to be insufficient to cause cervical cancer because there is a long incubation period between viral infection and tumor development. During this long incubation period, events such as viral integration into the host genome and virus-induced host chromosomal instability are thought to contribute to tumorigenesis (reviewed by Dell and Gaston^[8]). Cervical cancer is the second most common cause of cancer-related death in women worldwide, and in some developing countries, this disease is the leading cause of cancer-related mortality in women.^[9] In many developed countries, cervical-cancer screening programs have reduced the incidence of this disease.^[10] However, cervical cancer is still responsible for >13 000 deaths per year in the EU.^[11] Furthermore, cervical-cancer screening programs are costly and difficult to implement in developing countries. At present, malignant disease is typically treated by surgery and chemotherapy, and pre-cancerous lesions are typically treated by local excision or laser therapy.^[12] There are no treatments that specifically target HPV-transformed cells.

HR-HPV types also play a role in a proportion of cases of vulval carcinoma.^[13-15] Extensive local excision or laser therapy can be used to treat pre-invasive disease, vulval intraepithelial neoplasia (VIN) 1-3. However, recurrence is common.^[16]

Vulvectomy and radiotherapy are used in the treatment of vulval carcinoma. HR-HPV types are also thought to be involved in a variety of other malignant diseases, including some cases of oral cancer, skin cancer, tonsillar cancer, and cancer of the esophagus.^[17-21] Novel approaches to the prevention and treatment of cervical cancer and these other HPV-associated malignant lesions would be of great potential benefit.

1.1 Papillomavirus Biology

Papillomaviruses infect cells in the basal layer of the epithelial, and differentiation of the infected cells is required for completion of the viral life cycle (reviewed by Doorbar^[22]). The HPV genome is an 8000bp circular DNA molecule that is contained within an icosahedral protein coat to form the infectious particle. The viral genome encodes eight proteins (table I), although there are a number of splice variants. The early (E) genes encode proteins involved in DNA replication, transcription regulation, cell transformation, and virus assembly and release. The late (L) genes encode the viral capsid proteins. The genome also contains a non-coding region approximately 1000bp in length that is made up of transcription regulatory sequences and the viral origin of replication (for a review see Bernard^[23]).

The *E1* and *E2* genes encode DNA binding proteins that are required for viral replication. *E1* is a DNA helicase.^[24,25] *E2* is a transcription/replication factor that regulates viral gene expression by binding to four sites within the viral non-coding region and stimulates viral replication by recruiting *E1* to the origin or replication.^[26,27] *E2* is also thought to play a role in ensuring the equal segregation of viral genomes during host cell division.^[28,29] Alternative splicing produces a fusion protein in which the product of the *E8* open reading frame is fused to the C-terminal DNA binding domain of the *E2* protein. The resulting *E8E2C* fusion protein can repress HPV gene expression and downregulate viral replica-

Table I. Human papillomavirus proteins and their major functions

Viral protein	Function
E1	Replication factor – helicase
E2	Replication/transcription factor – origin binding protein
E4	Facilitates viral escape
E5	Oncoprotein – binds to growth factor receptors
E6	Oncoprotein – binds to TP53 and others
E7	Oncoprotein – binds to RB1 and others
L1	Major capsid protein
L2	Minor capsid protein

E = early; **L** = late; **RB1** = retinoblastoma tumor-suppressor protein; **TP53** = tumor-suppressor protein p53.

tion.^[30,31] The E4 protein is expressed late in the viral life cycle and disrupts the cellular cyokeratin network, possibly enabling virus release.^[32] The E5 protein is an integral membrane protein that interacts with cellular growth factor receptors and thereby promotes viral replication.^[33,34] L1 and L2 are the major and minor capsid proteins, respectively.

In the HR-HPV types, E6 and E7 are the predominant transforming proteins. E6 proteins from HR-HPV types bind to the cellular tumor-suppressor protein p53 (TP53) with high affinity, and in conjunction with a cellular protein known as E6AP (UBE3A), they target TP53 for degradation by the proteasome. This reduces the half-life of TP53 in HPV-infected cells, resulting in the partial inactivation of TP53-dependent functions.^[35-37] By degrading TP53, E6 inhibits activation of the cyclin-dependent kinase inhibitor p21 (CDKN1A) and the subsequent p21-induced growth arrest that normally occurs in response to signals that activate TP53.^[38,39] E6 also inhibits, but does not completely prevent, TP53-mediated apoptosis.^[40] The action of E6 on TP53 thereby generates a situation similar to that found in many spontaneous cancers in which TP53 is mutated.^[41,42] E6 proteins also interact with a number of other proteins that play roles in the regulation of the cell cycle and apoptosis. These include the tumor-suppressor protein 'human discs large' (DLG1)^[43] and BAK1 (BCL2 antagonist/killer 1) a pro-apoptotic protein,^[44] both of which are targeted for degradation by E6. Recent work has shown that E6 also interacts with the E2 protein, although the possible roles that this interaction plays in the viral life-cycle and tumorigenesis are not yet fully elucidated.^[45]

E7 proteins bind to the retinoblastoma tumor-suppressor protein RB1 and to the RB1-related proteins p107 (RBL1) and p130 (RBL2).^[46,47] The binding of E7 to these so-called 'pocket proteins' disrupts interactions with members of the E2F family of transcription factors.^[48] One consequence of the release of E2F family members is the activation of genes required for cell-cycle progression. This provides a suitable environment for viral replication within the infected cells.^[49] The binding of E7 to RB1 can also target this protein for degradation by the proteasome.^[50] The E7 proteins from HR-HPV types bind to pocket proteins with higher affinity than the E7 proteins from LR-HPV types, and this can account, in part, for the transforming activity of these E7 proteins.^[51-53] However, E7 is able to interact with a number of other proteins, including the TATA box-binding protein,^[54] interferon-stimulated gene factor 3 (ISGF3),^[55] and insulin-like growth factor-binding protein 3 (IGFBP3).^[56] IGFBP3 can inhibit cell proliferation and induce apoptosis. E7 targets IGFBP3 for degradation, and this could promote cell proliferation and inhibit apoptosis.^[56] Like E6, the E7 proteins have also been shown to interact with E2.^[57]

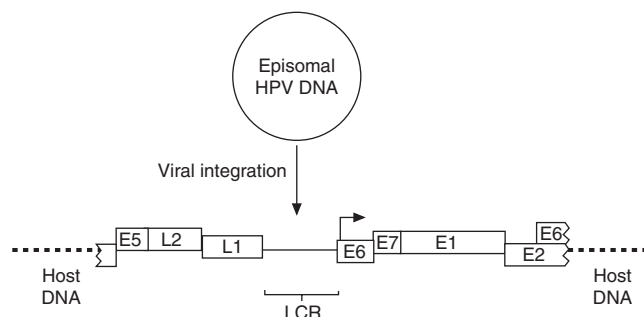


Fig. 1. Integration of human papillomavirus (HPV) DNA often occurs during tumorigenesis. The schematic shows the HPV genome before and after integration. Host chromosomal DNA is represented by the dashed line. The bent arrow indicates the early promoter and the direction of transcription. The viral genes are indicated in the integrated form (not to scale). **E** = early genes; **L** = late genes; **LCR** = the long control region/non-coding region.

1.2 Cell Transformation by HPV

In an infection, HPV genomes are present within the infected cells as episomes (free DNA circles). However, in the majority of cervical carcinomas, HPV DNA is integrated into the host genome (figure 1).^[58] In some cases, integrated viral DNA co-exists with episomal DNA.^[59,60] Following integration, viral particles can no longer be produced from the integrated HPV genomes, but continued expression of E6 and E7 prolongs the cell cycle and leads to the loss of effective DNA repair mechanisms.^[61] E6-E7 transcripts from integrated HPV genomes have increased stability compared with transcripts from episomal genomes, and integrated copies of the HPV genome have been shown to confer a growth advantage on the host cell.^[62,63]

The integration of HPV genomes is thought to occur at random positions within the host genome, although the sites of integration appear to correlate with chromosomal fragile sites in some cases.^[64-66] The viral genome has to be broken before integration can occur, and this is also presumed to occur at random positions within the episome. However, those integration events that are selected (those which result in a growth advantage to the host cell and are tumorigenic) often occur with the viral E2 gene. Some integration events occur within the E1 gene, but since E1 is upstream of E2 (see figure 1), these integration events also result in the loss of E2 expression.^[67] The loss of E2 expression (as well as E8E2C) is thought to result in the deregulated expression of E6 and E7, which in turn results in tumorigenesis. However, since E6 and E7 both bind to E2 protein, the loss of E2 may also affect the activities of these proteins within cells and not just their expression levels.^[45,57]

1.3 Prophylactic Vaccines

Vaccination against HPV could reduce the incidence of cervical carcinomas and other HPV-related tumors.^[68] However, it is difficult to generate HPV virions in cell culture, as viral replication is linked directly to the differentiation of the infected cell.^[69] Furthermore, even if HPV particles could be generated in large amounts using this approach, they would not be useful as a vaccine because the particles would contain the viral oncogenes. When over-expressed in cell culture, the HPV L1 capsid protein can self-assemble into virus-like particles (VLPs) that can induce high titers of neutralizing antibodies. VLPs have the potential to provide a safe subunit vaccine because they do not contain viral DNA. A phase I clinical study investigating the use of an HPV 18 L1 VLP found that the vaccine was well tolerated and highly immunogenic.^[70] Furthermore, vaccination with an HR-HPV 16 VLP can protect women against HR-HPV 16 infection.^[71] Recent work suggests that a bivalent recombinant VLP vaccine against HR-HPV 16 and 18 is effective against these HPV types for at least 4.5 years and appears to also offer protection against HR-HPV 45 and HR-HPV 31.^[72,73] The use of a quadrivalent recombinant VLP vaccine against LR-HPV 6, LR-HPV 11, HR-HPV 16, and HR-HPV HPV 18 protects against HR-HPV 16- and 18-associated CIN 2–3 as well as condylomata acuminata.^[74] This type of vaccine has the potential to protect against around 90% of genital warts and 70% of CIN 2–3 and cervical carcinomas. However, it is important to remember that there is a long incubation period between viral infection and the development of cervical carcinoma. None of the clinical trials conducted to date have been carried out over a sufficient time period and with adequate patient numbers to expect to see a significant number of cervical carcinomas in the untreated groups. Therefore, it is too early to be certain that these vaccines will reduce the incidence of cervical carcinoma. Furthermore, immunization using an effective vaccine is unlikely to be widely available within the medium term (5–10 years), and since there are >100 different types of HPV, any vaccine is very unlikely to be effective against all infections. In addition, the vaccination of children against what is effectively a sexually transmitted disease is likely to prove problematic, at least in some countries or populations.

1.4 Therapeutic Vaccines

The development of a prophylactic vaccine to protect against HPV infection is an attractive prospect; however, the neutralizing antibodies generated by such vaccines may not have the therapeutic capacity to clear established infections. Many women have existing HPV infections and many more will become infected before a prophylactic vaccine is available to all. Therapeutic

vaccines aim to bring about the regression of CIN 2–3 and/or to act as a treatment for cervical carcinoma. Therapeutic-vaccine development has focused mainly on the E6 and E7 oncoproteins because these are expressed in HPV-induced tumors. Clinical trials using a recombinant vaccinia virus expressing HR-HPV E6 and E7 showed that some patients with cervical carcinoma developed T cell immune responses after vaccination.^[75,76] Adenoviruses have also been used to express the HR-HPV E6 and E7 proteins for vaccination and used to infect dendritic cells (DC) for DC-based therapies.^[77,78] Several other studies have used proteins produced in bacterial cells, synthetic peptides, or DNA- or RNA-based vaccines that express E6 and/or E7.^[79–81] For example, mice vaccinated with DNA encoding E7-fused VP22 (a protein with novel intercellular trafficking properties that will be discussed further in section 3.2) generated a significant number of E7-specific cytotoxic T cells and showed a strong anti-tumor effect.^[82–84]

1.5 New Approaches to Treatment

Despite the promise of the vaccines described in sections 1.3 and 1.4, new approaches to the treatment of HPV-associated diseases are still required. Several potential drug targets have been identified; however, they are not reviewed in detail in this article. The E1 helicase is the only viral protein with enzymatic activity, and much effort has been devoted to the search for drugs that will act as specific E1 inhibitors. However, no suitable inhibitors of E1 are yet available. Drugs that target the interaction between E1 and E2 could also be effective^[85] but have yet to be developed for clinical studies. Since all HPV-transformed cells express the E6 and E7 oncoproteins, antisense technology has the potential to treat HPV-induced tumors by downregulating the expression of these proteins.^[86,87] However, it is not clear how HPV-targeted antisense molecules will be delivered *in vivo*. Cancer gene therapy might also provide methods for the treatment of HPV-induced disease, for example, over-expressing TP53 or RB1 in HPV-transformed cells (reviewed by Shillitoe^[88]). In the next section, we review the potential of the HPV E2 protein as a treatment for HPV-induced disease. Over-expression of the E2 protein can inhibit the growth of HPV-transformed cells.^[89–92] Furthermore, host antibodies against E2 might protect against future HPV infections.^[93] These properties of E2 suggest that the local delivery of this protein to the site of HPV infection might be a useful approach to treatment.

2. The E2 Protein

The HPV E2 protein is approximately 360 amino acids in length and consists of an N-terminal transcription activation/HPV replication domain and a C-terminal DNA binding domain, sepa-

rated by a highly variable region thought to act as a flexible hinge.^[26] E2 binds specifically to DNA sequences that correspond to the consensus 5'AACCGN4CGGTT3', where N represents any base.^[27] The HPV non-coding region contains four binding sites for E2 and numerous binding sites for cellular transcription factors. Sections 2.1 and 2.2 outline the host immune response to E2 and describe the effects of this protein on cell proliferation.

2.1 The Host Immune Response to E2

E2 is detectable in CIN 1 but is expressed at much lower levels in CIN 2–3.^[94] HR-HPV 16 E2-specific CD4+ T cells are present in healthy individuals, suggesting the presence of memory-type T cells,^[95] and in patients with pre-malignant disease, a T-helper-cell response towards E2 has been observed at the time of viral clearance.^[96] These observations suggest that the E2 protein might be useful as a potential therapeutic vaccine. Further evidence to support this proposal comes from the results of experiments performed using the cotton tail rabbit papillomavirus (CRPV). After vaccination with a recombinant adenovirus expressing the CRPV E2 protein, rabbits with an established CRPV infection showed a significant decrease in the number of papillomas, and those tumors that remained were reduced in size.^[97] Recent work has shown that a chimeric HR-HPV 16 VLP that includes E7 and E2 protein sequences has potential as a combined prophylactic and therapeutic vaccine.^[98] However, since E2 expression is lost in HPV-transformed cervical cancer cells, the use of E2 as a vaccine might be restricted to pre-malignant disease.

2.2 E2 and Cell Proliferation

Over-expression of the E2 protein can induce growth arrest and/or apoptosis in a variety of cell types.^[99,100] The bovine papillomavirus E2 protein and the HR-HPV 18 and HR-HPV 16 E2 proteins are able to repress the transcription of *E6* and *E7* in HPV-transformed cells.^[101,102] This results in growth arrest and the induction of cellular senescence.^[103,104] The over-expression of these E2 proteins increases the levels of p21 (CDKN1A), and this in turn induces cell-cycle arrest.^[104] The requirement for repression of E6 and E7 in E2-mediated growth arrest is perhaps best illustrated by the fact that exogenous E6 and E7 can rescue the growth of HPV-transformed cells expressing E2.^[105]

Several studies have demonstrated that the over-expression of E2 can also lead to the induction of apoptotic cell death.^[91,92,106] It is not known whether cells that originally escape E2-induced apoptosis undergo senescence or whether, alternatively, a population of cells undergo senescence and are protected from E2-induced apoptosis.^[104] Mutations within the DNA binding domain of E2 that block binding to DNA do not abrogate the

ability of E2 to induce apoptosis.^[106] In contrast, mutations within the N-terminal transcription/replication domain of E2 block the induction of apoptosis, which strongly suggests that this domain is required for the induction of apoptosis.^[107] Furthermore, the N-terminal domain alone can induce apoptosis when expressed in cells.^[107] These findings indicate that E2 is able to induce apoptosis via a pathway that is independent of its ability to regulate the transcription of *E6* and *E7*. This conclusion is further supported by experiments that have shown that the HR-HPV 16 and HR-HPV 33 E2 proteins can induce apoptosis in some HPV-negative cell types.^[106,108]

There is some debate surrounding the role of TP53 in E2-induced apoptosis. The induction of apoptosis by HR-HPV 18 E2 has been suggested to be independent of TP53 because BAX, a pro-apoptotic protein and a transcriptional target of TP53, is not activated following expression of HR-HPV 18 E2 in HPV-transformed cells.^[109] However, other studies have shown that the transcriptional activities of TP53 are not required for HR-HPV 16 E2-induced apoptosis.^[110] In addition, the HR-HPV 16 E2 protein does not induce apoptosis in TP53-null cells unless co-expressed with TP53.^[106] Furthermore, the HR-HPV 16 E2 protein has been shown to bind directly to TP53 both *in vitro* and in cells,^[111] and mutations that reduce the binding of this protein to TP53 inhibit E2-induced apoptosis in HPV-negative cells.^[110] Interestingly, the same mutations do not block the ability of E2 to induce apoptosis in HPV-transformed cells, presumably because the mutated E2 protein can still regulate expression of E6 and E7. In contrast, the HR-HPV 18 E2 protein has been reported to induce apoptosis in TP53-null cells, further suggesting that this E2 protein may induce apoptosis via an alternative, TP53-independent pathway.^[107] It cannot be ruled out that the E2 proteins of HR-HPV 16 and HR-HPV 18 use different mechanisms to induce apoptosis.

In summary, there are at least two mechanisms by which the E2 proteins can inhibit cellular proliferation (figure 2). In HPV-

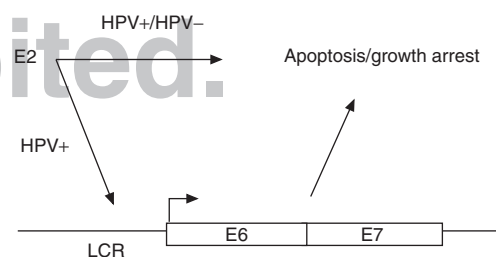


Fig. 2. The inhibition of cell proliferation by E2 proteins. In 'high oncogenic risk' human papillomavirus (HR-HPV)-transformed cells (HPV+), E2 can regulate transcription of *E6* and *E7* by binding to its sites within the long control region/non-coding region (LCR) of integrated HPV genomes and thereby induce growth arrest and apoptosis. E2 can also induce apoptosis independently of *E6* and *E7* in HPV-negative cells (HPV-) and HR-HPV-transformed cells.

transformed cells, E2 is able to bind to the viral non-coding region and repress the transcription of E6 and E7. This can induce both apoptosis and cell senescence. In addition, E2 can induce apoptosis via an HPV-independent pathway. This HPV-independent pathway can operate in HPV-transformed and non-HPV-transformed cells and appears to involve TP53.

3. The Delivery of E2 to HPV-Transformed and HPV-Infected Cells

The observations described above have led to the suggestion that E2 proteins could be used as a treatment for HPV-induced disease. This approach is particularly attractive given the fact that the E2 proteins would also generate an immune response that could protect against future infections. However, delivery of the protein to HPV-transformed cells and HPV-infected cells represents a major challenge.

3.1 Viral Delivery of E2

Recombinant viruses have the potential to deliver E2 proteins to target cells, with high efficiency. Vaccinia virus vectors producing the bovine papillomavirus E2 protein have been shown to inhibit the growth of HPV-transformed cells in nude mice.^[112] In a recent phase II clinical trial, a modified vaccinia virus expressing E2 was used to treat CIN 2–3 by direct injection into the uterus. In patients treated with the recombinant virus, the lesions were either eliminated or significantly reduced, and specific antibodies were generated.^[113] Vectors based on adenovirus, adeno-associated virus, or other non-integrating viruses could also be used to deliver E2. Recombinant viruses expressing E2 proteins or E2 derivatives have been produced by several groups.^[114–116] However, in most of these cases, transgene expression is transient and the immunogenicity of the vectors results in rapid clearance of the recombinant virus.^[117,118] Retroviruses could be used to express E2 over a longer time period. However, these vectors carry the risk of insertional mutagenesis. In addition, all of these virus-based delivery systems will leave a significant number of HPV-transformed or HPV-infected cells that are not infected by the recombinant vector. Unless the host immune response eliminates all of these untreated cells, the surviving cells will have the potential to regrow lesions, possibly many years after treatment is withdrawn.

3.2 Non-Viral Delivery of E2

Cell membranes present a major obstacle to the delivery of therapeutic molecules, and several non-viral vectors have been developed to address this problem. These non-viral approaches can deliver either nucleic acids or proteins. Liposomes have been the most commonly tested non-viral DNA delivery system used in

clinical trials.^[119,120] Liposomes are nonpathogenic, non-immunogenic, easy to manufacture, and could be used to deliver DNA encoding E2. However, liposomes have poor transduction efficiencies, and gene expression is transient. Gene guns could also be used to deliver DNA encoding E2. In this approach, a helium-powered gun is loaded with DNA-coated gold beads, which are then fired into cells.^[121] However, gene expression is transient and expression levels are generally low.

Cell-penetrating peptides are able to traverse the cell membrane and can be used to deliver therapeutic proteins (for a review see Deshayes et al.^[122]). Such cell-penetrating peptides include the HIV tat protein, a peptide derived from the *Drosophila* antennapedia protein (known as penetratin), and the herpes simplex virus type 1 (HSV-1) VP22 protein. VP22-E2 fusion proteins have been shown to enter mammalian cells *in vitro*.^[123] VP22 is encoded by the HSV-1 *UL49* gene.^[124] It is a 38 kDa protein with novel trafficking properties.^[125] VP22 is able to traffic between cells following transfection or microinjection.^[125,126] In cells that are actively producing VP22, the protein is exported via a Golgi-independent process. The exported VP22 can enter surrounding non-producing cells. Several proteins have been shown to acquire the ability to traffic between cells when fused to VP22, including green fluorescence protein,^[126] TP53,^[127–129] thymidine kinase,^[130,131] a peptide derived from BAK,^[132] and HR-HPV 16 E2.^[1,15,123] VP22 also appears to elicit an enhanced immune response towards the fused protein. For example, VP22-E7 fusion proteins produced a 50-fold increase in the number of E7-specific CD8 cytotoxic T cells over that seen with E7 alone.^[133] However, the use of VP22 has remained somewhat controversial. There have been claims that VP22-GFP fusions do not possess intercellular trafficking properties and that VP22 spread is an experimental artefact of fixation.^[134–136] However, VP22-GFP spread has been observed in living cells using time-lapse microscopy^[137] and flow cytometry.^[138]

A VP22-HR-HPV 16 E2 fusion protein (VP22-E2) has been shown to induce apoptosis in a variety of HPV-transformed cell lines.^[123] This fusion protein does not induce apoptosis in HPV-negative cells that lack functional TP53. This enables the production of VP22-E2 fusion proteins in COS-7 cells where TP53 is functionally inactivated by the SV40 T antigen.^[115,123] VP22-E2 proteins produced in COS-7 cells are transferred to non-producing cells in the absence of cell–cell contacts.^[115] If the recipient cells express functional TP53, the imported VP22-E2 can induce apoptosis. VP22-E2 proteins produced in bacterial cells can also enter mammalian cells and induce apoptosis.^[123] The spread of VP22-E2 proteins can be best observed in a 3-dimensional tumor model (figure 3a). When grown in organotypic culture conditions, HR-HPV-16-transformed SiHa cells mimic the 3-dimensional or-

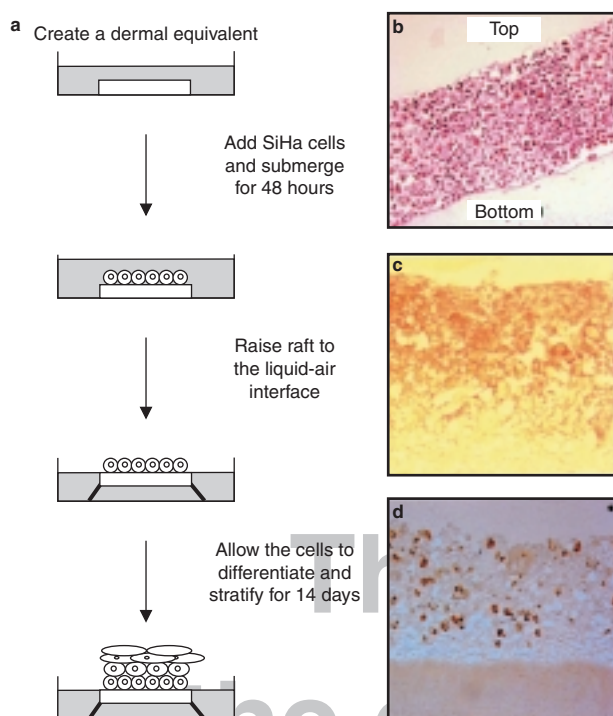


Fig. 3. VP22-E2 fusion proteins spread within a tumor model and induce apoptosis. (a) 'High oncogenic risk' human papillomavirus (HR-HPV)-transformed SiHa cells grown in organotypic culture for 2 weeks produce a 3-dimensional raft of cells that resemble a tumor. Adenoviruses expressing VP22-E2 fusion proteins can then be applied to the top surface of the raft. After 48 hours, the raft is embedded in paraffin and sectioned for immunohistochemical staining. (b) An untreated raft section stained with hematoxylin and eosin. (c) A treated raft section stained for VP22-E2 by immunohistochemistry. (d) A VP22-E2-treated raft section stained for apoptotic cells.

ganization of a cervical tumor (figure 3b). VP22-E2 fusion proteins can penetrate deep into these 3-dimensional rafts of cells (figure 3c) and induce apoptosis (figure 3d).^[115] These findings indicate that the local delivery of VP22-E2 fusion proteins could be used to treat cervical cancer and other HPV-associated diseases.

3.3 Targeting E2 to Tumor Cells and Other Challenges

A significant obstacle that must be overcome before E2 can be used as a therapeutic molecule stems from the fact that this protein can induce apoptosis in at least some HPV-negative cells.^[106] However, a mutated HR-HPV 16 E2 protein that fails to induce apoptosis in HPV-negative cells but which retains the ability to kill HPV-positive cells has been produced by site-directed mutagenesis.^[110] This mutated E2 protein shows reduced binding to TP53, and this may explain why it fails to induce apoptosis in HPV-negative cells. The mutated protein retains the ability to bind DNA and is able to induce apoptosis in HPV-transformed cells presumably by regulating the expression of E6 and E7.^[110] This

mutated E2 protein could also be delivered as a VP22-E2 fusion protein, and the hybrid has the potential to act as a selective therapeutic agent against HPV-associated conditions.

A second challenge is the production of a stable VP22-E2 protein that can be locally applied. Several lipid-based carrier systems that can deliver proteins into cells are available.^[139] In addition, a variety of hydrogels have been developed to stabilize and deliver recombinant proteins.^[140] Hydrogels are typically polysaccharides that form 3-dimensional networks that hold large amounts of water and take up proteins into this hydrophilic environment. Another approach that can be used to deliver proteins locally involves the production of an aerosol of the protein in the form of a dry powder.^[141]

Finally, it may be possible to increase the efficacy of E2 by combining this treatment with other drugs. The steroid hormones estrogen, 16 α -hydroxyestrone, and progesterone have all been shown to increase the levels of E2-induced apoptosis.^[142] The local application of E2 in combination with these or other molecules could also be a useful approach to treatment.

4. Conclusion

The topical application of E2 fusion proteins or viruses that express E2 could be useful in the treatment of a variety of HPV-associated diseases. Topical application of purified VP22-E2 proteins could be the preferred method of delivery, because this approach is inexpensive and does not require the large scale production of recombinant viruses. Although the E2 proteins can induce apoptosis in HPV-negative cells, this problem can be circumvented using mutated E2 proteins. As well as killing HPV-transformed and HPV-infected cells, E2 proteins generate immune responses that may prevent future infections.

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