Original

Enhancement of DNA Vaccine Potency Against Hamster Oral Papillomavirus-Associated Oral Cancer by Electroporation in vivo

Masato Jinno, Madoka Isomura, Nobuaki Sato, Yasuyoshi Torii, Waka Yoshida, Yoshihiko Sugita, Katsutoshi Kubo and Hatsuhioko Maeda

Department of Oral Pathology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan

(Received for publication, November 14, 2016)

Abstract: We previously developed a hamster oral papilloma virus (HOPV) tumor model. We also showed that immunization with a DNA vaccine consisting of naked plasmid DNA (pDNA) encoding the L1 region of the HOPV genome (pHOPV-L1) had a cancer suppressing effect in this model. Here, we investigated if the use of electroporation as a vaccine delivery system could enhance the effect of this vaccine. A pHOPV-L1 vaccine was generated and was inoculated (100 µg/animal) intramuscularly into hamsters (n=10 per group), who then underwent eight rounds of electroporation of 50, 100, or 200 V/cm (VE50, VE100, and VE200 group, respectively). Control groups (n=10 per group) were untreated (N), or were treated with electroporation alone (E50, E100, and E200 groups) or vaccination alone (V group). The animals then underwent carcinogenic treatment over the next 69 days, during which 9,10-dimethyl-1,2-benzanthracene (DMBA) was applied onto lingual mucosa and a lingual wound was created. Histopathological analysis of lingual tissue indicated that all of the animals in the N and E groups developed lingual carcinoma that was accompanied by koilocytosis in the squamous carcinoma lesions. In contrast, 4, 5, 7 and 9 animals in the V, VE50, VE100 and VE200 groups, respectively, showed no carcinoma lesions. All of the animals with carcinomas ultimately showed a decreasing trend in weight, as well as HOPV infection that was detected using real-time PCR analysis. On the other hand, all animals without carcinomas increased in weight over the entire course of the experiment and did not display HOPV infection. These data confirm that a vaccine consisting of naked pDNA from the L1 region of HOPV can suppress HOPV-associated cancer, and show that its cancer suppressing effects can be enhanced by low-voltage electroporation following inoculation. We conclude that low-voltage electroporation is a useful and safe delivery system for DNA vaccines and should be further exploited.

Key words: DNA Vaccine, Electroporation, HOPV, Oral cancer

Introduction

Vaccines are the oldest and one of the most effective medical technologies. Strategic vaccine design is now becoming possible with recent technological innovations, such as genetic recombination technology, chemical synthesis, and developments in immunology. New vaccines that are consequently drawing attention are DNA vaccines. DNA vaccines incorporate genes that express antigens of bacteria-derived circular DNA, referred to as “naked plasmid DNA” (naked pDNA), and induce a specific immune response to these antigens when administered in vivo. Compared to other types of vaccines developed to date, DNA vaccines are simpler and cheaper to produce. These vaccines are therefore being researched as new vaccines against infectious and allergic diseases, cancer, and other conditions, and their clinical application is progressing.

Human papilloma virus (HPV) infection is said to be the greatest risk factor for cervical cancer and precancerous lesions. HPV is also reported to play a part in oral cancer and head and neck cancer. HPV is, however, species-specific and does not infect animals other than humans. It is therefore impossible to directly show a relationship between virus-induced carcinogenesis and DNA vaccination by conducting in vivo infection experiments. We used the carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) to establish an experimental system capable of inducing epithelial dysplasia and squamous cell carcinoma in the oral mucosa of hamsters within a short period of 6-8 weeks. We also analyzed and determined the entire base sequence of the L1 region of HOPV genome by molecularly cloning the HOPV viral genome from this lesion. The results of this system are very similar to the pathology of HPV infection, which indicated that this HOPV tumor model is useful for analyzing in a short
period of time the role of HOPV in the papilloma virus infection-induced carcinogenic process. These results also revealed that HOPV plays an important role in this carcinogenic process. We therefore amplified each major gene in HOPV and inserted each of these genes into an expression plasmid to create naked pDNA for use as a DNA vaccine. We have already reported that inoculation of these naked pDNAs into the aforementioned HOPV tumor model to examine their effect revealed a cancer-suppressing effect when naked DNA encoding the L1 gene of the HOPV genome was inoculated into these model mice.

The way in which the DNA vaccine is taken up into the target cells is important in order for sufficient DNA to be taken up to induce continuous expression of the viral protein. Enhancement of the cancer-suppressing effects of DNA vaccines therefore requires examination of DNA vaccine delivery systems. Recently, electroporation, which uses the property of pulsed high voltage to create reversible pores in the cell membrane, has been developed as a method of inducing uptake of substances into cells. Drugs have reportedly been taken up into living cells at a high rate using such electroporation. Furthermore, application of pulsed high voltage to the cells of living tissue can create reversible pores in the cell membrane without damaging the cell, and gene-related substances such as DNA can also be inserted into living cells. Electroporation is therefore drawing attention as a useful method of enhancing in vivo transfer of naked pDNA into cells.

In this study, we examined if the cancer-suppressing effects of a DNA vaccine that was introduced into a HOPV tumor model could be enhanced by electroporation using various in vivo low-voltage pulsed loads. This study thus investigated the utility of in vivo low-voltage electroporation as a DNA vaccine delivery system.

**Materials and Methods**

**Creation of the DNA vaccine**

Based on the previously determined base sequence of the HOPV genome, we amplified the L1 region of the HOPV genome using the polymerase chain reaction (PCR) with the following primers, and then cloned this region by using the TOPO XL PCR Cloning Kit (Invitrogen, CA, USA).

The primers used were: 5’ cgggaattccg GATGGTGGATGGCAACCATCAGGCAAGCT 3’ (nt 5642-5668) and 5’ cgggaattccg ATTTGACATTCTTATTTACCGCTCTTA 3’ (nt 7133-7159). The primers contained a start codon (ATG underlined) and also have an added EcoRI site (underlined, lower case) at the 5’ end, and the cloned HOPV genome was also cut out with EcoRI. These DNAs were cloned into the pVAX1 vector (Invitrogen, CA, USA) and grown in E. coli. Subsequently, the pDNA containing the L1 gene was purified by the standard method using the QIAprep Spin Minipreptkit (QIAGEN, CA, USA) to create the naked pDNA vaccine (pHOPV-L1). The DNA base sequence was confirmed by BigDye Terminator Cycle Sequencing (Applied Biosystems, New Jersey, USA). The DNA vaccine (pHOPV-L1) was used in 100 µg doses dissolved in 100 µl of physiological saline.

**Inoculation method and electroporation treatment of the DNA vaccine, and the HOPV tumor model**

We performed vaccinations using the pHOPV-L1 DNA vaccine and performed in vivo low-voltage electroporation at three different voltages: 50 V/cm, 100 V/cm, and 200 V/cm. Eighty 3-week-old male golden hamsters (Japan SLC Inc., Hamamatsu, Japan) were used in the experiment and were assigned to the following eight groups (each containing 10 animals).

- Untreated group (N)
- Electroporation alone groups: 50 V (E50), 100 V (E100), and 200 V (E200)
- Vaccination alone group (V)
- Vaccination with electroporation groups: 50 V (VE50), 100 V (VE100), and 200 V (VE200)

In the untreated group (N), 100 µg of pVAX1 alone dissolved in 100 µl of physiological saline was inoculated into the left quadriceps of the animals. In the vaccination alone group (V), the animals were inoculated with pHOPV-L1 (100 µg/100 µl). The pHOPV-L1 vaccine was inoculated into the left quadriceps of the animals at a dose of 100 µg dissolved in 100 µl of physiological saline. In the electroporation alone groups (E50, E100 and E200), pVAX1 (100 µg/100 µl) was inoculated after which electroporation was performed. In the vaccination with electroporation groups (VE50, VE100 and VE200), the same pHOPV-L1 vaccine (100 µg/100 µl) was inoculated, after which electroporation treatment was performed. Electroporation treatment involved loading the animals with low-voltage pulsed stimulation (at 50, 100, and 200 V/cm) eight times for 20 ms at 1 Hz with a square electroporator (CUY21, Tokyo, Japan) and by using a tweezer-type electrode while the animals were under Nembutal anesthesia 3 minutes after inoculation with pHOPV-L1 or pVAX1. After these treatments, the same carcinogenic treatment was performed as that in the HOPV tumor model. Carcinogenic treatment involved applying a 0.5% acetone solution of DMBA (Sigma-Aldrich Japan, Tokyo, Japan) onto the tongue 3 times a week for 8 weeks, excising 2 mm of the lingual tip, and then applying the lingual tip with DMBA daily for 13 days in accordance with the method described by Maeda et al. One day after completing this carcinogenic treatment, the animals were sacrificed under anesthesia and examined for suppression of carcinogenesis. Additionally, all experimental animals underwent weight measurements every week during the experiment.

**Examination of suppression of carcinogenesis**

After carcinogenic treatment, lingual tissue was excised from all the animals and histopathologically examined. The status of
Figure 1: Weight changes in the untreated and electroporation alone groups

HOPV infection was also examined at the time of lingual excision and upon completion of the experiment by using real-time PCR.

To extract DNA for HOPV examination, lingual tissue was harvested from the animals at the time of lingual excision and upon completion of the experiment, fixed in formalin and embedded in paraffin. The paraffin-embedded tissue was then used to create 10 continuous 4-µm sections and the first section was stained with hematoxylin and eosin stain (HE) to confirm the presence of histological features of squamous cell carcinoma and normal epithelium. Thereafter, the remaining nine sections were used to extract DNA. Using laser capture microdissection (Laser Capture Microdissection System, Archturus Engineering Inc., Mountain View, CA, USA), 200 cells per sample (squamous cell carcinoma or normal epithelium) were harvested with transfer film and used to extract DNA.

DNA was extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Tokyo, Japan) according to the product protocol. The amount of extracted DNA was also confirmed by using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

Next, the HOPV-L1/β-actin value was determined using real-time PCR as follows. The DNA expression level of the HOPV L1 region and β-actin as the control were examined using the following primers and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in the ABI 7700 Sequence Detection system (Applied Biosystems).

Table 1: Anti-tumour effects against hamster lingual carcinoma to administration of hamster oral papillomavirus L1 gene vaccine with or without Electroporation.

<table>
<thead>
<tr>
<th>Group/ pDNA dose (mg)</th>
<th>Total number of hamsters</th>
<th>Number of hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage (V/cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>E 50</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>E 100</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>E 200</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>VE 50</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>VE 100</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>VE 200</td>
<td>200</td>
<td>10</td>
</tr>
</tbody>
</table>
HOPV-L1 primers:
5’ GGAGACTCAGGGGACTTGAAATAGGACGT 3’ (nt 5932-5960)
5’ GACCATCACTGGATAGTAGGGATCCACT 3’ (nt 6534-6561);
β-actin (golden hamster) primers
5’ A TATTGAGAACA TCGTTCCCC 3’ (nt 1417–1427)
5’ CCACAAGTAGTCAAGGCAGGT 3’ (nt 2082–2102).

These oligonucleotide primers were synthesized by Sigma-Aldrich Japan (Tokyo, Japan) and were used in all real-time PCR tests. Amplification of HOPV-L1 was done with 40 cycles of 95°C for 10 min, 94°C for 10 min, 50°C for 1.5 min and 72°C for 2 min, followed by 72°C for 10 min, and β-actin was amplified with 35 cycles of 95°C for 10 min, 94°C for 2 min, 55°C for 1.5 min, and 72°C for 2 min, followed by 72°C for 5 min.

The animals in this study were used in accordance with the guidelines for animal experiments of the School of Dentistry of Aichi Gakuin University.

Results

Histopathological examination of suppression of carcinogenesis
Carcinogenesis was seen in the tongues of all animals in the untreated (N) and electroporation alone groups (E50, E100, and E200) (Table 1). While temporary weight gain was observed in all of the animals in these groups, these animals ultimately showed a decreasing trend in weight (Fig. 1). Histopathologically, the animals also showed koilocytosis (Fig. 3 inset), which was in well-differentiated squamous cell carcinoma with visible cancer pearls (Fig. 3).

In the vaccination alone group (V) inoculated with pHOPV-L1, four of the 10 animals showed suppression of carcinogenesis (Table 1). These animals that did not show carcinogenesis showed an increase in weight throughout all carcinogenic treatment periods (Fig. 2). Histopathological examination revealed an epithelium with wound healing after excision of the lingual tip, and fibrous connective tissue hyperplasia beneath this epithelium (Fig. 4).

In the vaccination with electroporation groups (VE50, VE100 and VE200), five, seven, and nine out of the 10 animals in the respective groups showed suppression of carcinogenesis (Table 1). Furthermore, these animals that did not show carcinogenesis also showed an increase in weight throughout all carcinogenic treatment periods like those in the V group (Fig. 2) but showed no histological abnormalities. However, the animals in the V, VE50, VE100 and VE200 groups that showed carcinogenesis
showed a temporary weight gain like those in the N, E50, E100 and E200 groups, but ultimately showed a decreasing trend in weight (Fig. 2). Histopathologically, these animals had the same squamous cell carcinoma as the animals in the N group.

**HOPV infection**

HOPV infection had increased after carcinogenic treatment in all the animals that showed squamous cell carcinoma in the N, E50, E100 and E200 groups (Table 2). The same increase in HOPV was also seen in the animals that showed carcinogenesis in the V, VE50, VE100, and VE200 groups; however, almost no change in the amount of HOPV compared with that seen at the start of the experiment was observed in the animals that showed no carcinogenesis (Table 2).

The above results proved that the cancer-suppressing effect of the vaccine was strongest in the VE group that underwent both DNA vaccination and *in vivo* low-voltage electroporation, and that the suppressing effect was strongest at a voltage of 200 V, followed by 100 V and 50 V.

**Discussion**

Papilloma virus causes proliferative lesions of stratified squamous epithelium and is attracting attention as a major cause of squamous cell carcinoma in the human cervix, head and neck region, oral cavity, and other regions of the body. We were able to create pre-cancerous and cancerous lesions in the hamster lingual mucosa at a high rate within a short period of time by applying DMBA onto lingual mucosa and creating a lingual wound. We previously reported detecting development of koilocytosis, immunohistological papilloma virus-positivity, and papilloma virus-like particles by electron microscopy in these lesions, which are features of papilloma virus infection. These findings indicate that HOPV infection is closer to HPV infection than it is to HPV infection. HPV infection. These similarities with the pathology of HPV infection suggested that this HOPV tumor model could be very useful in developmental research into DNA vaccines based on HPV infection, which is why we used this model in our study.

The HOPV in this study is similar to HPV in that it has circular double-stranded DNA with a chain length of approximately 7,700 bp as its genome, and is reported to have both early gene groups (E region: E1, E2, E4, E6, and E7) and late gene groups (L region: L1 and L2). Considering the similarities with HPV, HOPV infection that occurs in the epithelial cells of hamster tongues could ultimately integrate the E6 and E7 genes and trigger cell proliferation. However, initial infection of HOPV normally exists in the form of an episome. The HOPV genome is thought to later integrate into the infected cell’s DNA in response to some kind of host factor, such as a decrease in immune capacity or a carcinogen, and consequently forms a lesion. In the carcinogenic process in the HOPV tumor model in this study, carcinogenesis is thought to have occurred when HOPV was integrated into the host DNA as a result of applying a carcinogen onto the tongue and healing of the lingual wound. This integration of HOPV into the host DNA also suggests that HOPV is an important factor in carcinogenesis in this tumor model.

DNA vaccines have several advantages over conventional vaccines, including the ease of their design and manufacture, their ability to readily induce specific antibodies and cytotoxic T cells, and their excellent thermal stability and storability. DNA vaccines are also capable of inducing both cellular immunity and humoral immunity. Plasmid DNA, which is the main part of a DNA vaccine, is usually introduced into a living body by intramuscular injection using a syringe or by subcutaneous...
inoculation using a gene gun. In the case of an intramuscular injection, plasmid DNA is taken up into muscle cells and antigen-presenting cells within the muscle tissue, and, once the biosynthesized protein has been broken down into the appropriate length, it is presented by MHC class I molecules and activates CD8-positive T cells\(^1\). Meanwhile, protein secreted and leaked from muscle cells and antigen-presenting cells is taken up by other antigen-presenting cells, broken down into the appropriate length, and presented by MHC class II molecules to stimulate CD4-positive T cells, thereby mainly inducing Th1 cells\(^1\). In addition to inducing antibody production by stimulating B cells through interactions between cytokines and B cells, helper T cells also support the induction of cytotoxic T cells. Furthermore, a portion of the T cells and B cells prepare for re-infection of pathogens as memory cells. Currently, other methods of vaccine administration such as administration via the mucosal system are being examined in addition to intramuscular injections and gene guns. However, the immunological mechanism of action of DNA vaccines is not yet properly understood. The way in which DNA administered to the living body acts on the immune system and the method by which the effects of DNA vaccines are induced are particularly unclear.

Recently, the right-handed double helical structure of DNA (B-DNA) has been found to act as an endogenous vaccine adjuvant by activating the innate immune system via the intracellular enzyme (signaling molecule) known as TANK-binding kinase 1 (TBK1). This signal for innate immune system activation has been identified as essential for the effect of DNA vaccines\(^26,27\). Furthermore, of the effects of DNA vaccines, TBK1-dependent innate immune activation of immune cells such as dendritic cells is important for the production of antibodies, while TBK1 activation in non-immune cells such as muscle cells that have taken up DNA is important for the activation of cellular immunity by T cells\(^23\). However, progress is yet to be made in clarifying the reason why DNA vaccines work, including the immunological mechanism of action.

In this study, we performed intramuscular inoculation with a DNA vaccine of naked pDNA from the L1 region of HOPV (pHOPV-L1) and examined its cancer-suppressing effects. The results of our investigation are presented in Table 1 and show that no tumor formation occurred in four of the 10 animals in the vaccination alone group. These four animals showed an increase in weight throughout all carcinogenic treatment periods (Fig. 2). They also showed normal wound healing in a histopathological examination (Fig. 4). HOPV-L1/β-actin values assessed using real-time PCR were also lower in the lingual tissue of these animals. However, all the animals in the electroporation alone and the untreated groups that received just the vector ultimately showed a decreasing trend in weight gain (Fig. 1) and also histopathologically showed squamous cell carcinoma accompanied by koilocytosis (Fig. 3). Furthermore, these animals had high HOPV-L1/β-actin values by real-time PCR analysis. These results suggest that the use of naked pDNA from the L1 region of HOPV as a DNA vaccine can suppress the onset of HOPV-related squamous cell carcinoma. These results are similar to those reported by Maeda et al.\(^9\) and indicate the potential usefulness of DNA vaccines in the prevention of HPV-related squamous cell carcinoma of the cervix and oral cavity.

There are various theories regarding which region of the HOPV genome should be used as a DNA vaccine\(^28,29\); however, the L1 region has reported efficacy as a prophylactic DNA vaccine in the prevention of HPV infection\(^30\). The L1 and L2 genes in HPV are late expression genes that code for two types of structural proteins, L1 and L2, which are capsid proteins\(^9\). The use of L1 and L2 regions as DNA vaccines promises humoral immunity to these capsid proteins as antigens. However, the ratio of the L1 protein to the L2 protein per virus particle is thought to be 30:1\(^9\). This ratio suggests that a stronger immune effect can be obtained by using the L1 region as the DNA vaccine. Moreover, although when creating a DNA vaccine, the use of the E6 and E7 regions should offer a greater anti-tumor effect, these E6 and E7 genes are cancer genes and pose safety issues when used in their original form to create DNA vaccines. All of the above suggests that the L1 region is the more effective and safer region for use as a DNA vaccine.

To enhance the effects of DNA vaccines, it is important to consider how DNA vaccines are taken up into cells and how the viral protein is continuously expressed. In subcutaneous inoculation, the DNA particles are fired at high speed by a gene gun and taken up into the cells\(^30\). This intracellular uptake is enhanced by using electroporation\(^31\). The use of electroporation in intramuscular inoculation was similarly recently reported to enhance the effect of inoculation\(^32\).

In this study, we used in vivo low-voltage electroporation when inoculating a HOPV tumor model with pHOPV-L1 as the DNA vaccine and found that the cancer-suppressing effect was greater than in the vaccination alone group that underwent no electroporation (Table 1). Furthermore, this cancer-suppressing effect was strongest in the order from 200 V, 100 V, then 50 V. HOPV-L1/β-actin values assessed using real-time PCR were also lower in animals that showed no carcinogenesis in the DNA vaccination group in which low-voltage electroporation was used. These results suggested that in vivo low-voltage electroporation is a useful delivery system for DNA vaccines.

To date, electroporation has been used as a DNA vaccine delivery system in a study in which a DNA vaccine that coded for prostate-specific antibodies in mice was used to enhance cell immunity against these antibodies\(^33\), and in a study in which a DNA vaccine for a foot-and-mouth disease virus in mice was used to enhance the antibody titer of the virus\(^34\). Both of these studies reported strong effects of the vaccines. Another study reported
that a DNA vaccine coding for the E6/E7 fusion protein of HPV18 that was introduced into mice using electroporation enhanced cellular immunity against an E6/E7 fusion protein. These reports also demonstrate that the effects of DNA vaccines are enhanced by using electroporation. However, these studies did not examine cancer suppression using actual animal experimental models, but rather examined the effects of DNA vaccines on cellular immunity. Our study is therefore the first to analyze cancer suppression by a DNA vaccine using in vivo low-voltage electroporation.

Furthermore, while voltages ranging from 100 V/cm to 3,500 V/cm have been used in electroporation, many studies have used low voltages that do not cause side effects such as burn injury in living bodies. In this study, we also used low voltages of 50 V/cm, 100 V/cm, and 200 V/cm, which all resulted in cancer suppression. Of these voltages, we found that the DNA vaccine cancer-suppressing effect is enhanced from 50 V/cm upwards. Low-voltage in vivo electroporation that does not cause side effects such as burn injury in living bodies is therefore very safe and shows promising effects as a delivery system for DNA vaccines used in HPV infection in humans. In any event, the method of DNA vaccine delivery is very important and delivery needs to be done taking into consideration the type of infectious virus and the lesion. Our findings also suggested the need to conduct further study into HOPV DNA vaccine delivery systems and to examine methods of creating and using DNA vaccines using other major HOPV genes.

The aforementioned results demonstrated that in vivo low-voltage electroporation is a useful and very safe delivery system for DNA vaccines. Going forward, DNA vaccines for cancer treatment that use the major HOPV genes E6 and E7 will need to be developed in addition to DNA vaccines used in HPV infection in humans. The results of this study could also contribute to the clinical application of DNA vaccines against HPV infection in humans.

Acknowledgements
This research was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, under a Grant-in-Aid for Scientific Research C (Grant No. 15K11031).

Conflict of Interest
The authors have declared that no COI exists.

References


