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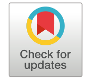
Original Article

Human Papillomavirus Type 16 Based L1, L2, E6, and E7 Peptide Microspheres Induce Encapsulated Peptide Mixture Specific Cytotoxic T Lymphocytes and Tumor Regression in a Murine Model of Cervical Cancer



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Abstract

Background and objectives: Infection with HPV16, a high-risk human papillomavirus (HPV), can cause cervical cancer in humans. These infections carry a high risk of morbidity and mortality globally in females. This study aimed to conduct an *in vivo* comparison of Poly (D,L-lactic-co-glycolide) (PLGA)-encapsulated peptide mixture nanoparticles and PLGA microspheres as delivery systems for vaccines.

Methods: PLGA polymers were used to form microspheres for a therapeutic vaccine against cervical cancer. The target antigens were the L1 and L2 capsid proteins and the E6 and E7 oncoproteins from HPV16. These antigens were selected based on their immunogenicity, allergenicity, and toxicity. We predicted epitopes for cytotoxic T lymphocytes (CTLs) and helper T lymphocytes. In our investigation of CTL epitopes, we employed synthetic chimeric PLGA microsphere peptides, consisting of multiple H-2Db-restricted HPV16 peptides, coupled with other immune-potentiating adjuvants as predicted by our work.

Results: H-2Db-restricted HPV16 peptides, when administered subcutaneously, enabled CTLs to eliminate *in vitro* TC-1 tumor cells expressing E6 and E7 of HPV16. Additionally, *TC-1* cells protected C57BL/6 mice against *in vivo* challenges. To address this problem, peptide-based vaccines, which are among the most effective vaccine systems, have been extensively studied. Combining peptide-based vaccinations with microsphere peptide mixture particles and delivery technologies enhances their efficacy in stimulating cellular immune responses and eliminating tumor cells.

Conclusions: This approach may provide a potential therapeutic candidate vaccine based on microsphere-encapsulated peptides for the prevention of cervical cancer caused by HPV.

Introduction

One type of malignant tumor connected to the cervix is cervical cancer.[1] The human papillomavirus (HPV) is a virus associated with different types of malignancies and causes deadly cancer. It has been observed in approximately 99.7% of cases of cervical cancer worldwide.[2] Approximately, 91% of deaths from HPV-associated cancer are attributable to cervical cancer. It is the second most common disease among women aged 15 to 44 and the fourth most common malignancy overall.[3,4] Additionally, almost 70% of cervical malignancies are caused by HPV genotypes 16 and 18.[5] E6 and E7 are important viral proteins that play a key role in eliciting oncogenesis in infected cells, leading to uncontrolled cell division and unrestrained telomerase activity, which result in the progression of cervical cancer.[6] In addition to the cervix, HPV can also lead to cancer in the penis, vagina, oropharynx, and vulva.[1,7] Squamous cells typically make up the outer layer of the cervix, while columnar gland cells make

Keywords: Cervical cancer; Microsphere peptide mixture; Human papillomavirus (HPV); Therapeutic vaccine; Cytotoxic T lymphocyte response; CTL.

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up the interior layer. In particular, the first infection may result in dysplasia, called *in situ* adenocarcinoma. Various methods such as surgery, radiation therapy, and/or chemotherapy have been used for the treatment of cervical cancer.[8] Without the risk of serious side effects, vaccination against HPV infection is a promising new alternative therapeutic.[9] Vaccinations use live-attenuated pathogenic antigens generated from pathogenic bacteria or viruses to activate the immune response and induce neutralizing antibodies.[10] The targeted cellular immune responses are generated by novel vaccinations for the treatment of cancer.[11] By employing sequences of immunogenic antigens against HPV-associated proteins, peptide-based vaccines are an excellent option for immunization, as they can effectively eradicate HPV infections and elicit cellular immune responses. Peptide-based vaccines are advantageous due to their ability to elicit cellular immune responses and eradicate HPV infections, making them an ideal option for effective vaccination. Peptide-based vaccines can be quickly obtained through straight forward methods and generally exhibit enhanced stability during storage and transportation, as well as bioavailability.[12] The stimulatory effect on the immune system is mediated by microspheres due to their surface-anchored targeting moieties, hydrophobicity, size, and charge.[13] Improved nanoparticles can more successfully transfer associated antigens to antigen-presenting cells (APCs) initiating a robust and effective immune response. In addition, certain cytokines (interleukin (IL)-4, IL-10, tumor necrosis factor (TNF)-β and interferon (IFN)-γ) are secreted during the adaptive immune response. These cytokines activate cellular signaling pathways that regulate immune responses against specific antigens by binding to particular receptors on the surface of immune cells.[14] When phagocytized or endocytosed cells are displayed on APCs, they break the incoming pathogens (or antigens) into tiny peptides. Major Histocompatibility Complex (MHC-I or MHC-II, allowing CD8 or CD4 cells to detect them, subsequently stimulating humoral and/or cellular defense against invasive infections.[11] Targeting intracellular antigens, cancer immune therapies, and vaccinations aim to elicit cellular immune responses. In particular, vaccination candidates must be delivered as endogenous antigens that trigger a cell-mediated immune response when they are displayed by MHC I to $CD8^+$ T-lymphocyte cells after being broken down in the host cytoplasm by the proteasome. HPV-infected cells are cleared by cell-mediated immunity.[15] Adjuvants such as PLGA microspheres were utilized to test the possibility of eliciting immunological responses against HPV16-associated malignancies. The introduction of a mixture of cytotoxic T lymphocyte (CTL) peptides in combination with microsphere adjuvants led to immunogenicity against *TC-1 cell*-induced tumors *in vivo*. Generally, APCs phagocytose and transport the peptide combination (L1, L2, E6, and E7) into draining lymph nodes. The usage of adjuvant enables the peptide microspheres to be released continuously over several days or even weeks. We have demonstrated that HPV16 chimeric CTL peptide immunization with an H-2Db-restricted antigen, in conjunction with adjuvant microspheres, generated an immune response specific to peptides and demonstrated *in vivo* anticancer efficacy against *TC-1 cells* that expressed HPV16 E6 and E7 in C57BL/6 mice. Before being applied in a clinical setting, these techniques must still be optimized for efficacy, safety, and specificity. We believe that a targeted gene therapy approach will benefit cervical cancer patients as long as the programmable structure and functions are further understood.

Materials and methods

We designed *in silico*, chimeric peptides $L1^{165–173}$ (9 mer), $L2^{108–120}$ (13 mer), $E6^{48–57}$ (10 mer), and $E7^{48–57}$ (10 mer) of HPV16, using the algorithmic prediction software CTLpred (available at: http://www.imtech.res.in/raghava), each containing a CTL epitope. Target epitopes were predicted using a neural network along with the physicochemical properties of the antigen.[16] The small peptides (9–13 mer) were formed into a single large chimeric peptide associated with a linker of two glycines to form $\{E6^{48–57}\}GG\{E7^{48–57}\}GG\{L1^{165–173}\}GG\{L2^{108–120}\}$ (48 mer), E6–E7–L1–L2 containing multiple CTL epitopes. The chimeric peptide conformation was analyzed using PyMOL software. These peptides were synthesized commercially by Genscript Corporation, USA (purity 95% by high performance liquid chromatography, dissolved in phosphate-buffered saline (PBS), and stored at −70°C until use.

Adjuvants

PLGA was used for microsphere formation and obtained from Boehringer Ingelheim (Ingelheim, Germany).[17] The solvent double evaporation method (water/oil/water) was used to encapsulate the synthetic peptides of HPV16 L1, L2, E6, and E7 in the PLGA. The percentage entrapment of each peptide in the PLGA microsphere was determined by double solvent extraction followed by a BCA protein estimation assay (Pierce).

Mice and cell lines

C57BL/6 (H-2b) female mice (5–6 weeks old) were subcutaneously (s.c.) immunized with the encapsulated PLGA peptide microsphere mixture (200 µg/100 µL PBS per mouse). Control mice received the same volumes of PBS & PLGA microsphere. Each group of mice received two booster shots at 10-day intervals. Within 7–10 days after the last booster shot, the mice were euthanized, and their spleens were removed for *in vitro* work. The American Type Culture Collection (USA) provided the tumor cell line *TC-1*, created by retrovirally transducing lung fibroblasts of C57BL/6 origin with the HPV16 E6/E7 virus and the c-Hras *oncogene*. These cells were cultivated in RPMI-1640 medium with 10% (v/v) fetal calf serum, supplemented with nonessential amino acids (2 mM), and G418 (0.4 mg mL^{-1}) at 37°C with 5% CO_2.

In vivo tumor regression assay

To achieve *in vivo* tumor regression, viable *TC-1 cells* (1×10^5 cells in 100 µL PBS per mouse) were injected s.c. into the left flank of C57BL/6 mice on the first day of the experiment. On day 8, each peptide microsphere was injected s.c. with 200 µg of the PLGA microsphere mixture, followed by two booster shots on days 18 and 28. Tumor size was measured every 3–5 days for 35 days using calipers once palpable tumors (7–10 days post-TC-1 inoculation) formed.[18] Tumor volumes greater than 2,000 mm^3 resulted in the death of the mice.

Enzyme linked immunosorbant assay (ELISA)

As previously mentioned, ELISA was performed on polystyrene modules with a flat bottom.[19] To ascertain the degree of peptide-specific antibodies in vaccinated mice, 100 µL of a peptide microsphere cocktail (containing L1, L2, E6, and E7) was coated with 0.5 µg of pure encapsulated PLGA antigens for 16 h at 4°C. The antigens were diluted in carbonate-bicarbonate buffer (0.05 M, pH 9.6). After washing the plates with Tris buffer saline-trixon (TBS-T) (20 mM Tris, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20) and blocking the unoccupied sites with 150 µL of 5% fat-free milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4–6 h, serum samples (diluted 1:100) were added in triplicate and incu-

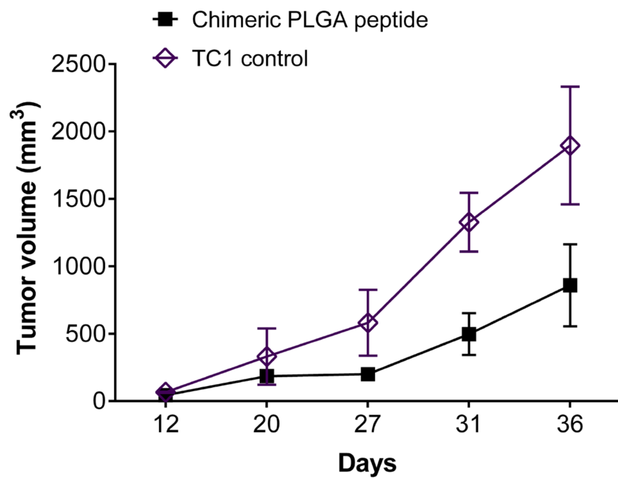

Fig. 1. Tumor challenge and *in vivo* tumor growth with TC-1 tumor cells. Mean tumor volume ± SD (mm^3) from mice (n = 8) immunized with chimeric peptide microspheres was compared to the respective control mice (injected with PBS along with microspheres) on the indicated days following the tumor challenge. *$P < 0.05$. PBS, phosphate-buffered saline; PLGA, Poly (D,L-lactic-co-glycolide); SD, standard deviation.

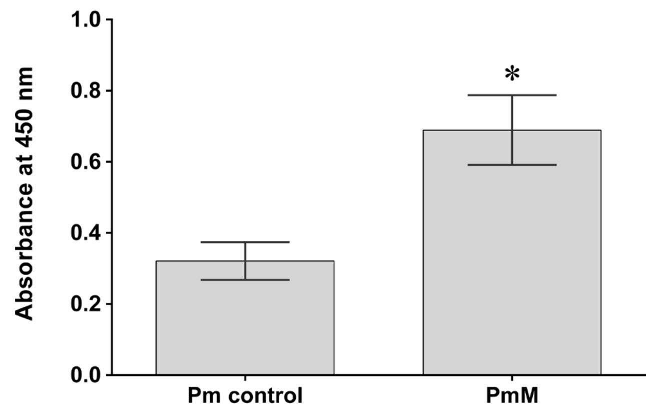

Fig. 2. ELISA for serum IgG levels of microspheres and peptide mixture encapsulated microsphere wells were coated with the respective antigens. Data are reported as mean ± SEM; *$P < 0.05$. ELISA, enzyme linked immunosorbant assay; IgG, immunoglobulin G; PM, PLGA Microsphere; PmM, PLGA encapsulated peptide mixture.

bated at 37°C for 1 h.[20] The plates were washed four times using TBS-T. To assess the bound antibodies, goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase-conjugated antibody (diluted 1:2,000; Invitrogen) was added and incubated at 37°C for 1 h. Following a final wash, the enzyme-substrate TMB (Sigma) was applied, and immunoreactivity was measured using an ELISA plate reader (Biotek) to detect absorbance at 450 nm.

Lymphocyte proliferation assay

Following *in vitro* stimulation with chimeric encapsulated PLGA peptide microspheres (10 µg per well), lymphocyte proliferation was measured as previously reported.[20] Splenocytes (5×10^5 cells per well) were aliquoted at 100 µL in 96-well plates with RPMI-1640 supplemented with 10% (*v/v*) Foetal Calf Serum, IL-2 (10 Uml^{-1} recombinant-mouse), and 2-mercaptoethanol (0.05 µM). Promega Corp. USA's Cell Titer was utilized to gauge lymphocyte proliferation from various cohorts by (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) assay. Cytotoxicity percentage was computed as follows:

$$\%\ \text{Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

Cytolytic activity against TC-1 tumor cells

As previously reported,[15] effector-mediated cytolysis of *TC-1 cells* was analyzed using the CytoTox 96 Non-Radioactive test kit (Promega Corp. USA) and a lactate dehydrogenase assay.[21]

Estimation of cytokines

To conduct a lymphocyte proliferation experiment, 96-well plates containing 10 µg of chimeric encapsulated PLGA peptide microspheres were used to cultivate splenocytes from both the control and immunized groups. After 48 h, supernatants were collected for the cytokine test. The amounts of cytokines were measured using Endogen mouse *IFN-γ, IL-2, IL-10,* and *IL-4* ELISA kits (Pierce Biotechnology, Inc. USA).

Statistical analysis

The Student's *t-test*, Mann-Whitney U test, and Wilcoxon signed-rank test were used for statistical analysis. The population-averaged model of the generalized estimating equation was used to examine the tumor regression percentage. *P-values* of ≤ 0.05 were considered significant.

Results

Regression of tumors against post-TC-1 by Chimeric encapsulated PLGA peptide microsphere

The encapsulated PLGA chimeric peptide microsphere was able to regress E7-related tumors after *s.c.* injection of the *TC-1 cell line* on day 1, for the tumor regression test. Tumor development was observed from the 7th day onward. The encapsulated PLGA chimeric peptide was injected post-tumor assay to assess tumor regression. The *TC-1 cell line* showed tumor progression to 1,895.64 mm^3 but the encapsulated PLGA peptide mixture showed tumor regression to 858.34 mm^3. The data showed a significant increase in tumor regression compared to controls, being 54.72% for the encapsulated PLGA peptide mixture (Fig. 1).

Immunoglobulin G responses by subcutaneous immunization of HPV16 encapsulated PLGA peptides (L1, L2, E6 & E7) cocktail

Subcutaneous immunization of the PLGA microsphere peptide cocktail was found to induce antibodies in sera capable of binding to the HPV16 PLGA peptide mixture (L1, L2, E6, and E7) as shown in Figure 2. Serum was collected on day 27 after the first immunization. Encapsulated PLGA peptide-specific IgG antibodies in serum were higher compared to the control PLGA microsphere in mice. The serum IgG level of the PLGA peptide cocktail was significantly higher compared to the IgG level of the control PLGA microsphere.

Chimeric encapsulated PLGA peptide mixture induced lymphocyte proliferation

Harvesting of splenocytes for *in vitro* stimulation with chimeric peptides of PLGA showed a statistically significant increased proliferation ($P \leq 0.05$; Mann–Whitney U test) compared to control microspheres. In response, the lymphocyte proliferation, measured

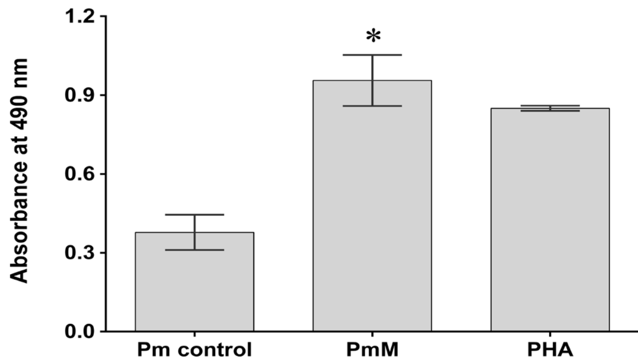

Fig. 3. *In vitro* lymphocyte proliferative response and antigen-induced cytotoxic T lymphocyte generation in C57BL/6 mice immunized with chimeric peptide. Proliferation of lymphocytes was determined by MTT assay. Data are reported as mean ± SEM; **P* < 0.05. MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide); PHA, phytohaemagglutinin; PM, PLGA microsphere; PmM, PLGA encapsulated peptide mixture.

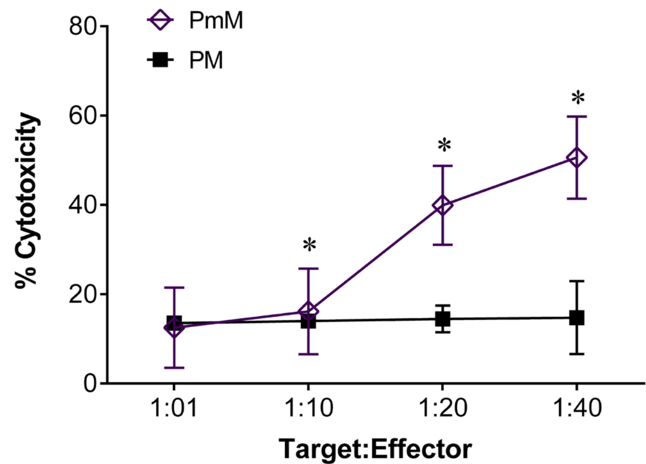

Fig. 4. *In vitro CTL* cytotoxicity of *TC-1* tumor cells: *CTLs* were measured by LDH estimation at 7 days after the last booster. Pooled effector splenocytes of mice immunized with encapsulated peptide mixture and the control group (n = 5 in each group) were co-cultured with target *TC-1* cells, and cytolysis of the latter was determined. Data are expressed as mean ± SE of three separate experiments; **P* < 0.05, Wilcoxon rank sum test. CTL, cytotoxic T lymphocyte; LDH, Lactate Dehydrogenase; PM, PLGA Microsphere; PmM, PLGA encapsulated peptide mixture.

at OD_{490} by MTT assay, yielded mean values (stimulation indices ± SEM) of 0.856 ± 0.104, 0.331 ± 0.082, and 0.819 ± 0.01, respectively, with PLGA peptide mixture and control PLGA microsphere (Fig. 3). When paired analysis (i.e., unstimulated vs. stimulated lymphocytes from the immunized group) was performed, the proliferation level was shown to be considerably ($P \leq 0.05$; Mann–Whitney U test or Wilcoxon signed-rank test).

Cytotoxicity for peptide mixture against TC-1 cells

After *in vitro* stimulation, viable effector cells from the vaccinated and control groups with the PLGA peptide combination were evaluated for their cytotoxic efficacy against *TC-1 cells.* Figure 4 illustrates the percentage difference in cytotoxicity resulting from effector cell-mediated lysis of *TC-1 cells*. Using the PLGA microsphere peptide mixture, the percentage cytotoxicity was 70.50%, which was significantly greater than the target: effector ratio of unstimulated effector cells from control groups at 1:40.

Th-1 type response against chimeric peptide by cytokines estimation

In comparison to the control group, stimulated splenocytes exhibited reduced levels of *IL-10* and moderate amounts of *IL-2* and *IL-4*. Additionally, they displayed greater levels of *IFN-γ*. Immunized groups had noticeably increased levels of *IFN-γ* secretion. A notably elevated release of *IL-2* was seen in the microsphere. PLGA microspheres were used as an adjuvant, and among the Th-2 subtype, significant levels of *IL-10* and *IL-4* were found in the culture supernatant in the PLGA peptide combination (Table 1).

Discussion

Peptide antigens can be more specifically and effectively delivered *via* microspheres, which also enhance immunogenicity stability and durability. Controlling the biophysical features of microspheres such as size, shape, and surface qualities is relatively easy and can influence the immune responses against the corresponding antigen.[22] One important factor influencing antigen retention and the immunogenicity of peptide-based vaccines is the nanostructure manufacturing method.[23] Generally, microspheres possess immune-stimulating qualities that facilitate antigen delivery and enhance absorption and recognition by antigen-presenting cells.[17] Moreover, antigens prepared or encapsulated in microspheres are resistant to enzymatic breakdown. Characteristics such as longer half-lives and increased permeability through barriers, such as mucosal tissues, are also observed in microspheres within the body.[24] An appealing method to activate the immune response against high-risk HPV varieties associated with infections is therapeutic vaccination.[25] Targeting antigens against HPV that are constitutively expressed in HPV-associated cancers is crucial for therapeutic vaccination,[26] particularly for HPV16 L1, L2, E6, and E7, which play roles in tumor etiology, cellular transformation, and virus replication.[27] The production of peptide vaccines is simple, safe, and restricted to a particular major histocompatibility complex. Initially, we identified unique immunogenic epitopes of HPV16 antigens using software, which overlapped with immunodominant epitopes for $L1^{165–173}$,[28] $L2^{108–120}$, $E6^{48–57}$,[29] and $E7^{49–57}$ in murine (H-2Db) CTLs.[30] Combining these epitopes into a single large chimeric peptide enhances antigen absorption and synchronizes the activation of antigen-presenting cells. We also

Table 1. Cytokine estimation of immunized culture supernatant splenocyte and control group

S.No	Groups	Cytokines (pg/mL)			
		IFN-γ	***IL-2***	***IL-4***	***IL-10***
1	Control PM	46.71 ± 0.73	45.31 ± 0.93	72.31 ± 0.002	2.59 ± 0.63
2	PmM	1,234.46 ± 125.30	74.07 ± 53.06	187.34 ± 105.77	12.86 ± 3.13

For n = 6 mice, the data are presented as mean ± SE in pg/mL. *P*-values were determined using the Mann-Whitney U test to compare cytokine levels between the immunized and control groups. *P* < 0.05 was considered significant. IFN, interferon; IL, interleukin; PM, PLGA Microsphere; PmM, PLGA encapsulated peptide mixture.

investigated how adjuvants and delivery methods affect these peptide antigens. The E6 and E7 proteins, epitopes of cytotoxic T lymphocytes found in capsid proteins L1 and L2, are known to elicit weakened immune responses in cells. Strong models have been developed to select HPV16 E6 and E7 oncoproteins for creating peptide-based anticancer vaccines.[31] By using cutting-edge adjuvants and delivery methods, the production of *CTLs* and antibodies induced by peptide immunogens can be significantly improved. Mice were immunized using Freund's complete adjuvant, demonstrating immunogenic potential for peptides Q19D and Q15L from HPV16 E6 and E7 oncoproteins ($E6^{43–57}$ and $E7^{44–62}$, respectively).[32] Subcutaneous vaccination with an H-2Db-restricted HPV16 chimeric PLGA-encapsulated peptide produced peptide-specific *CTL*-mediated cytolysis for *TC-1* tumor cells *in vitro*. Additionally, *C57BL/6* mice were protected against an *in vivo* challenge by *TC-1 cells*. With the tested microsphere adjuvant, this peptide effectively induced *CTLs* that showed good effectiveness. Furthermore, these adjuvants activated splenocytes, triggering peptide-specific lymphocyte proliferation to varying degrees, while showing lower levels of *IL-10* and moderate levels of *IL-2, IL-4,* and *IFN-γ* in the culture supernatant. *In vivo* testing revealed that both the PLGA microsphere adjuvant and chimerically encapsulated peptide had similar anticancer effectiveness, likely due to the ongoing immune system-tumor cell conflict during the malignant transformation process, where tumors develop defense mechanisms to evade immunosurveillance, particularly with persistence.[33] Therefore, lengthy overlapping peptides may be used in future peptide microsphere vaccines to increase the variety of antigenic determinants and decline the barrier for restriction of major histocompatibility complexes. Successful T-cell immune responses have been demonstrated with overlapping peptides in both preclinical animal models and human clinical trials.[34,35]

Conclusion

Cervical cancer, caused by HPV, affects millions worldwide, yet there is currently no effective therapeutic vaccine. In this study, we attempted to develop a multi-epitope cervical cancer vaccination. We have developed a peptide-based microsphere candidate vaccination for HPV16-related infections and tumors, showing significant antigen-specific therapeutic effects in a mouse model of cervical cancer. Thus, our preclinical findings provide a solid foundation for future clinical research.

Acknowledgments

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Funding

None.

Conflict of interest

None.

Author contributions

Study concept and design (MAK, MAR); acquisition of data (MAK); assay performance and data analysis (MAK, KA, MAR); drafting of the manuscript (MAK, KA); critical revision of the manuscript (MAR); supervision (MAK). All authors have made a significant contribution to this study and have approved the final manuscript.

Ethical statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Indian Council of Medical Research (ICMR) New Delhi, India. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Uttar Pradesh, India (AECN: 234/18/JN/AMU). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Data sharing statement

Not available additional data.

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