A Murine Model for the Effects of Pelvic Radiation and Cisplatin Chemotherapy on Human Papillomavirus Vaccine Efficacy

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Abstract

Therapeutic human papillomavirus (HPV) vaccines for cervical cancer depend on a competent immune system to be effective. However, cancer patients are often found to be immunosuppressed, which could be attributable to prior radiation, chemotherapy, or the tumor burden itself. This study investigated whether pelvic radiation or cisplatin treatment affected the efficacy of an HPV vaccine and how long these effects lasted. Mice were given pelvic radiation, 2 Gy/day to a total dose of 45 Gy, or 5 mg/kg/week of cisplatin for 3 weeks. Mice were then immunized with an HPV-16 peptide vaccine between 0 and 16 weeks after their treatment. An ELISPOT analysis revealed that a reduced level of peptide-specific, IFN-γ-producing spleen cells was present in immunized mice treated previously with pelvic radiation or cisplatin compared with immunized mice that had not been treated. However, when mice were challenged with HPV-16-expressing tumor cells, immunized mice developed no tumors, regardless of prior treatment, whereas immunocompromised mice did develop tumors. Our results suggest that pretreatment with pelvic radiation or cisplatin alone does not prevent the induction of an effective immune response by a peptide vaccine. These data will have important implications for immunotherapeutic treatment of pretreated cancer patients, especially in the adjuvant setting when immunosuppression by tumor burden would be low.

Introduction

It is estimated that 12,800 new cases of cervical cancer will be diagnosed in the United States in the year 2000, and 4,600 deaths will occur over the same time period because of this disease (1). The incidence and mortality of this disease have decreased >40% in the United States in the last 25 years, largely because of cytological screening with the Pap test. Worldwide, however, Pap testing is not uniformly implemented, and death attributable to cervical cancer remains a major health problem.

HPV infection rates in cervical cancer are now reported to be 99.7%, and the presence of HPV is regarded as a necessary cause (2). Because expression of the E6 and E7 proteins of high-risk HPV subtypes is required for maintenance of the malignant phenotype (3), these proteins have become attractive targets for therapeutic HPV vaccines (4).

Standard treatment of cervical cancer includes pelvic radiotherapy and chemosensitization with a cisplatin-based regimen (5–9). Published immunotherapy trials against cervical cancer using HPV-16 E7 peptides have enrolled patients with recurrent or advanced disease, most of whom were heavily pretreated with radiation therapy, chemotherapy, and/or surgery. Because many of these women were found to be immunocompromised, these trials have demonstrated rather poor immune responses (10–13). Reasons for the observed immune suppression could include the radiation and chemotherapy pretreatment that these women had received.

Local radiotherapy can result in lymphopenia, attributable to lethal effects on both the lymphocytes circulating in the radiation field and to the stem cells within the local marrow (14). Studies of radiation therapy in cancer patients in general and in cervical cancer patients specifically have demonstrated a decreased number of circulating T cells, a decreased CD4+:CD8+ ratio, a persistent decrease in naive T cells compared with memory T cells, and a decrease in the T-cell proliferative responses. There also seems to be an inverse relationship between tumor load and immune status that may be reversible when the tumor burden is diminished (15–20).

Systemic chemotherapy is well known to cause neutropenia; however, significant T-cell depletion can occur as well. With the initiation of chemotherapy, T-cell numbers can decline rapidly, with a preferential depletion of CD4+ cells compared with CD8+ cells. Recovery of B-cell and CD8+ cells usually takes place by 3–6 months after the completion of chemotherapy. However, frequently a CD4+ lymphopenia persists for years. There is a preferential rise in CD45RO+ cells over CD45RA+ cells, resulting in a prolonged deficiency in naive T cells (21–23).

How these factors affect the efficacy of a therapeutic tumor vaccine is unknown. Therefore, we developed an animal model to specifically investigate the effect of either pelvic radiotherapy

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or cisplatin chemotherapy on the host’s ability to respond to a vaccine. This model studies peptide vaccination in tumor-free mice; therefore, any immunosuppressive effects of an in situ tumor is avoided. We also performed these experiments to study the duration of the immunosuppressive effect, if present, to gain insight into the optimal time after pelvic radiotherapy or cisplatin chemotherapy to start vaccination.

Materials and Methods

Mice. C57Bl/6 (B6) mice were obtained from Harlan (Indianapolis, IN). All mice were 6–7 weeks of age at the initiation of experiments. All experiments were approved by the Joint Institutional Animal Care and Use Committee of Loyola University Chicago.

Radiation Experiments. All mice were placed in an appropriate plastic animal holder, and their hind legs were fixed, thus stabilizing their pelvises in the holder. The mice were then placed in a lead block with a 1-cm opening superiority and inferiorly over the pelvis and placed into a gamma irradiator (Gammacell). Radiation was delivered from a 137cesium source using a two-field, anteroposterior/posteroanterior technique. Mice received a calculated dose of 203 cGy daily, 5 days/week, to total a dose of 4466 cGy in 22 fractions. This dose and schedule were chosen because of their similarity to patient treatment. Groups of seven mice received their peptide vaccination 0, 1, 2, 4, 8, 12, or 16 weeks after completing radiation.

Chemotherapy Experiments. Cisplatin has been used in mouse models at a dose between 5 and 8 mg/kg given either i.v. or i.p. These doses have been well tolerated and able to shrink xenografted human tumors implanted into mice (24–27). The cisplatin dose used in our mouse model, 5 mg/kg, is at the lower end of this range, relatively similar to the doses used for chemosensitization in humans. Because i.p. dosing of cisplatin results in plasma concentrations similar to i.v. dosing (28), we split the mice when they became 80–90% confluent by detaching with trypsin/EDTA. Prior to tumor challenge, cells were allowed to grow for 36–48 h and were used between passages 9 and 19. In preparation for injection, cells were detached with trypsin/EDTA, washed three times in HBSS (Sigma), and brought to a concentration of 2 × 10^6 cells/ml in HBSS. Mice were challenged 2 weeks after immunization with 200 μl of this cell suspension, or 5 × 10^5 cells, injected s.c. in their right flank. The same investigator monitored all mice for tumor growth for 45 days after injection of the tumor cells.

Peptide-specific ELISPOT Assay. Two to three mice/group in each experiment were not challenged with C3 tumor cells and instead were sacrificed by cervical dislocation 14–16 days after immunization. Spleens were harvested immediately under sterile conditions. A single cell suspension was made by forcing spleens through a 200-μm filter. Cells were suspended in 50% IMDM, 40% FCS, and 10% DMSO (Sigma) and stored in liquid nitrogen until assays were performed.

ELISPOT assays were performed on these previously frozen splenocytes. On day 1, IFN-γ antibody (1 mg/ml stock; PharMingen) was diluted with PBS to 10 μg/ml. Fifty μl of the diluted antibody were applied per well to a 96-well MultiScreen-HA plate (Millipore, Bedford, MA), which was incubated overnight at 4°C.

On day 2, the plates were washed and blocked with IMDM. Splenocytes were thawed and brought to a concentration of 1 × 10^6 live cells/ml. Samples from each mouse were run in triplicate. Cells were incubated at a concentration of 2 × 10^4 cells/well in 200 μl of IMDM with 20 units/ml interleukin 2 and 10 μg/ml RAHYNIVTF overnight at 37°C with 5% CO_2_.

On day 3, plates were washed and incubated for 2 h at room temperature with biotinylated IFN-γ antibody at a concentration of 5 μg/ml in 50 μl. After washing, avidin-alkaline phosphatase (Sigma) was added at a concentration of 1.25 μg/ml in 50 μl and incubated for 2 h at room temperature. After washing, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Promega Corp., Madison, WI) was added in 50 μl to each well. The plate was incubated for 15 min precisely, and the reaction was stopped by rinsing the plate with water. Spots/well were counted, and the number of IFN-γ-producing cells per 1 × 10^6 splenocytes was calculated.

Statistical Analysis. In the tumor challenge experiments, comparison of the rates of tumor protection in experimental groups to that in the negative control group who received no vaccine was made using Fisher’s exact test. Comparisons of the number of IFN-γ-producing cells among the groups were conducted using both parametric and nonparametric techniques. Because each provided similar results, the parametric approach using ANOVA is presented. When the ANOVA was statistically significant, the Duncan test was performed to compare individ-
To investigate the immunosuppressive effects of pelvic radiotherapy on vaccine efficacy, we performed the following experiments.

For the radiation experiments, groups of seven mice received pelvic radiation in a manner similar to cervical cancer patients, at a dose of 2 Gy/day to a total dose of 45 Gy. They were then vaccinated s.c. with an HPV-16 E7 peptide vaccine in IFA between 0 and 12 weeks after completing radiation. Two mice/group were sacrificed, and their splenocytes were harvested. An ELISPOT assay was performed to look at the numbers of RAHYNIVTF-specific, IFN-γ-producing cells among groups. These statistics were run including the positive, but not the negative, control as an independent group. All statistical analyses were performed using the SAS software. In all cases, an adjusted two-sided α level of 0.05 was considered statistically significant.

Results

To investigate the immunosuppressive effects of pelvic radiotherapy or cisplatin chemotherapy on the number of peptide-specific IFN-γ-producing cells induced by this strong vaccine, we performed the following experiments.

For the radiation experiments, groups of seven mice received pelvic radiation in a manner similar to cervical cancer patients, at a dose of 2 Gy/day to a total dose of 45 Gy. They were then vaccinated s.c. with an HPV-16 E7 peptide, RAHYNIVTF, emulsified in IFA, between 0 and 16 weeks after completing treatment. Two mice/group in each experiment were sacrificed, and their splenocytes were harvested. An ELISPOT assay was performed to look at the numbers of RAHYNIVTF-specific, IFN-γ-producing cells within each group. The mean numbers of splenocytes/animal did not differ between groups (data not shown).

As seen in Fig. 1, there were differences in the number of peptide-specific, IFN-γ-producing cells among groups. It appears that pelvic radiotherapy decreases the number of peptide-specific cells stimulated in response to our vaccine in most groups when compared with the untreated, vaccinated control group.

To investigate the effects of cisplatin on vaccine efficacy, groups of 14 mice received three weekly doses of i.p. cisplatin chemotherapy. Two mice/group were sacrificed, and their splenocytes were harvested. An ELISPOT assay was performed to look at the numbers of RAHYNIVTF-specific, IFN-γ-producing cells. Combined results from two independent experiments are shown. Negative controls were not vaccinated; positive controls received no radiation but were vaccinated (P = 0.14, ANOVA). Bars, SD.

Results

Effect of pelvic radiation on the induction of HPV-16 E7-specific, IFN-γ-producing cells. Three mice/group in each experiment were sacrificed, and their splenocytes were analyzed by ELISPOT for peptide-specific, IFN-γ-producing cells. Combined results from two independent experiments are shown. Negative controls were not vaccinated; positive controls received no radiation but were vaccinated (P = 0.04, ANOVA). * significantly lower peptide-specific cells than positive control.

Fig. 1 Effect of pelvic radiation on the induction of HPV-16 E7-specific, IFN-γ-producing cells. Groups of mice received 2 Gy/day pelvic radiation to a total dose of 45 Gy. They were then vaccinated s.c. with an HPV-16 E7 peptide vaccine in IFA between 0 and 12 weeks after completing radiation. Two mice/group were sacrificed, and their splenocytes were analyzed by ELISPOT for peptide-specific, IFN-γ-producing cells. Combined results from two independent experiments are shown. Negative controls were not vaccinated; positive controls received no radiation but were vaccinated (P = 0.14, ANOVA). Bars, SD.

Fig. 2 Effect of i.p. cisplatin on the induction of HPV-16 E7-specific, IFN-γ-producing cells. Groups of mice received three weekly doses of 5 mg/kg i.p. cisplatin. They were then vaccinated s.c. with an HPV-16 E7 peptide vaccine in IFA between 0 and 12 weeks after completing chemotherapy. Three mice/group were sacrificed, and their splenocytes were analyzed by ELISPOT for peptide-specific, IFN-γ-producing cells. Combined results from two independent experiments are shown. Negative controls were not vaccinated; positive controls received no cisplatin but were vaccinated (P = 0.04, ANOVA). * significantly lower peptide-specific cells than positive control.
vaccine efficacy as determined by the rejection of a C3 tumor, the remaining 11 mice in each group noted above that received cisplatin chemotherapy were vaccinated with the E7 peptide vaccine between 0 and 12 weeks after completing treatment. The mice were challenged 2 weeks after the vaccination with the C3 tumor cells, and monitored regularly for tumor growth.

Fig. 4 illustrates the results of these tumor challenge experiments. Seventy-seven % of mice that were not vaccinated; positive controls received no radiation but were vaccinated. All vaccinated mice were significantly better protected against the tumor challenge than the unvaccinated negative control mice ($P = 0.00012$, Fisher’s exact test). ctrl, control.

Discussion

In a recent clinical trial, a peptide vaccine was used to immunize women with documented, HPV-16-associated, high-grade cervical and vulvar intraepithelial neoplasia. Although evidence of depressed immune function was demonstrated even in this population with preinvasive disease, as seen by a decreased T-cell receptor $\gamma$-chain expression, the majority of patients had a detectable immune response against the peptide after vaccination. This response was demonstrated by an augmentation of T-cell cytokine release, an increase in T-cell cytotoxicity, and a high rate of regression of the cervical intraepithelial neoplasia or vulvar intraepithelial neoplasia lesions (33). Most importantly, this study demonstrates that a peptide vaccine strategy is capable of inducing a cellular and clinical immune response.

Clinical trials in advanced or recurrent cervical cancer patients using the same peptide as in the trial noted above were not nearly as successful in inducing an immunological or clinical response (10–13). Women in these trials demonstrated a high rate of immunosuppression, as evidenced by low baseline CD4+ counts, low absolute lymphocyte counts, and decreased response to common skin test antigens. This immune suppression could be an impediment to the application of a therapeutic HPV vaccine. The majority of these women had a large tumor burden, which itself is associated with impaired immune function (20), and most of them have been pretreated with a combination of radiation therapy, chemotherapy, and surgery. How each of these factors contributed to poor immune function, and specifically one’s ability to respond to a vaccine, is difficult to determine.

Our series of experiments were designed to specifically study the effect of the treatment with either radiation or chemotherapy on vaccination efficacy. By vaccinating tumor-free mice, the potential immunosuppressive effects of an in situ tumor on the development of a tumor-directed immune response were avoided. Our results demonstrate that both pelvic radiotherapy and cisplatin chemotherapy may result in fewer numbers of E7 peptide-specific, IFN-$\gamma$-producing cells when compared with untreated, vaccinated controls; however, this did not affect the overall efficacy of the vaccine to reject a tumorigenic tumor challenge. There may be a threshold of specific T-cell numbers above which protection is afforded. If this is the case, our therapy did not decrease the T-cell numbers below this threshold, and therefore the vaccine efficacy was not affected in the tumor challenge experiments. It is also possible that the IFN-$\gamma$-producing cells that we measured by ELISPOT, thought to represent peptide-specific CTLs, are not an appropriate surrogate marker for vaccine efficacy.

Standard therapy for cervical cancer patients now includes pelvic radiation and cisplatin-based chemotherapy. In February
1999, the National Cancer Institute widely publicized the results of five randomized clinical trials (5–9) that demonstrated a 30–50% improvement in survival with the use of cisplatin-based chemotherapy concurrently with primary radiation therapy. Cisplatin doses used in those experiments are sensitizing doses and not full therapeutic doses. We chose a low therapeutic dose for the mice to mimic this clinical situation. Pelvic radiation given to the mice in our study is very similar in dose and schedule to the external radiation received by patients. For these reasons, we feel that this model closely represents a patient treatment course. We purposefully did not combine the two modalities to look at each individually.

Our data suggest that pretreatment with pelvic radiation or cisplatin alone does not prevent the induction of an effective immune response. Despite reduced numbers of IFN-γ-producing cells in most of the treated, immunized groups compared with the untreated, immunized controls, these mice were able to mount a peptide-specific immune response that rejected growth of the tumor. There was a significant decline in peptide-specific, IFN-γ-producing cells when the vaccine was given within 2 weeks of completing cisplatin therapy. In designing further human vaccine clinical trials, it would likely warrant waiting the relatively brief period of 3–4 weeks after completing chemotherapy before beginning the vaccine protocol.

Our experiments were performed in one animal model using tumor-free mice. This allowed us to isolate the immunological effect of each treatment from the other and from the immunosuppressive effect of an in situ tumor, making our experiments most applicable to immunotherapy used in the adjuvant setting. In this case, patients have already undergone primary chemoradiation, have no clinically detectable residual disease, but are at high risk for persistent microscopic disease and subsequent relapse and would be excellent candidates for adjuvant immunotherapy. Although combination chemoradiation may produce a more clinically significant immune suppression, our data suggest that potential immunosuppression by pretreatment with local radiation or cisplatin chemotherapy may not prevent the induction of an immune response to a vaccine in cancer patients. These results pave the way for a peptide vaccination trial for cervical carcinoma in a Phase II adjuvant setting.

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