

Therapeutic Synergy of Human Papillomavirus E7 Subunit Vaccines plus Cisplatin in an Animal Tumor Model: Causal Involvement of Increased Sensitivity of Cisplatin-Treated Tumors to CTL-Mediated Killing in Therapeutic Synergy

Sung Hwa Bae,¹ Young-Ja Park,² Jae-Bok Park,³ Youn Seok Choi,⁴ Mi Suk Kim,⁵ and Jeong-Im Sin²

Abstract Purpose: The goal of this study was to investigate the therapeutic potentials of combining chemotherapy with human papillomavirus (HPV) E7 subunit vaccines in an animal tumor model and to determine the underlying therapeutic mechanisms.

Experimental Design: Animals bearing HPV E6/E7-expressing tumors were treated intratumorally with a selected cytotoxic drug, cisplatin, twice at 1-week interval and s.c. with E7 subunit vaccines thrice at 1-week interval. Tumor chemoimmunoresponse was measured by tumor size. Ag-specific CTL activities and tumor histology were checked in mice under treatments. Apoptosis, *in vivo* T-cell subset depletion, adoptive CTL transfer, and tumor regression were used to determine the mechanisms for antitumor therapeutic effects.

Results: Combined therapy using cisplatin plus E7 subunit vaccines improved cure and recurrence rates of tumors and long-term antitumor immunity dramatically more than single therapy alone. In particular, both components of E7 subunit vaccines were required for induction of Ag-specific CTL as well as therapeutic synergy when combined with cisplatin. This therapeutic synergy was abrogated by depletion of CD8⁺ T cells *in vivo* and was concomitant with histologic changes (such as heavy infiltration of lymphocytes and reduced tumor cell density). Finally, the increased sensitivity of cisplatin-treated tumors to CTL-mediated killing was found to be responsible for therapeutic synergy.

Conclusions: E7 subunit vaccines plus cisplatin mediate antitumor therapeutic synergy through the increased sensitivity of cisplatin-treated tumors to CTL-mediated killing. Moreover, E7-based therapeutic vaccines have the potential to improve chemotherapy in patients with cervical cancer.

Cervical cancer of the uterus is caused mainly by infection with human papillomavirus (HPV; ref. 1). Presently, HPV virus-like particle-based prophylactic vaccination against HPV infection is expected to reduce cervical cancer incidence. In women already infected with HPV, however, therapeutic vaccines are one promising option for control of cervical cancer incidence. To date, HPV E6 and E7 proteins have been a major target for immune-based therapeutic strategies against HPV-derived cervical cancers as they are expressed constantly in cervical

cancer cells (2, 3). In particular, a variety of E6- or E7-targeted vaccine types have been tested preclinically and in humans. These include peptide vaccines (4–6), DNA vaccines (7–10), recombinant proteins (11–13), dendritic cell-based vaccines (14–17), and viral/bacterial delivery of vaccines (18–20). These studies have shown the importance of T-cell responses, particularly CTL in clearing cervical lesions in humans or tumors in animals. In our recent findings, tumor burden is inversely related to E7 subunit vaccine-induced antitumor therapeutic efficacy (21). For instance, E7 subunit vaccines were able to cure tumors of a smaller size (2 mm in diameter) but not larger tumors (6–8 mm in diameter). This prompted us to evaluate whether E7 subunit vaccines might be able to cure tumors of a larger size when combined with chemotherapy. Furthermore, previous studies using other tumor model systems have shown antitumor combination effects when integrating dendritic cell-based vaccines with cytotoxic drugs (22, 23). However, no studies have yet been reported on the mechanisms as to how this occurs.

Presently, concurrent chemoradiation therapy has been the current 'standard of care' for locally advanced cervical cancers. The previous literature has suggested a large benefit of concurrent chemoradiation for survival, progression-free survival, and local and distant control rates in cervical cancer patients (24). However, recurrence of the disease has been

Authors' Affiliations: Departments of ¹Hematology-Oncology, ²Microbiology, ³Pathology, and ⁴Obstetrics and Gynecology, Catholic University of Daegu, Daegu, Korea and ⁵Department of Obstetrics and Gynecology, Flushing Hospital Medical Center, Flushing, New York

Received 7/25/06; revised 10/9/06; accepted 10/16/06.

Grant support: Basic Research Program of the Korea Science and Engineering Foundation grant R01-2005-000-10158-0.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Jeong-Im Sin, Department of Microbiology, School of Medicine, Catholic University of Daegu, 3056-6, Daemyung-4-Dong, Namgu, Daegu, 705-718, Korea. Phone: 82-53-650-4338; Fax: 82-53-651-8327; E-mail: jsin1964@hanmail.net.

© 2007 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-06-1838

problematic. It has been thought that growth of tumor cells and response to conventional therapy modalities (surgery, chemotherapy, and radiotherapy) are influenced by the host immune status. In particular, CTL responses to E6 and E7 are more commonly detectable in HPV-16-positive women without cervical intraepithelial neoplasia than in HPV-16-positive women with cervical intraepithelial neoplasia (25), suggesting the importance of CTL in disease protection. Similarly, CTL has a protective effect in squamous intraepithelial lesion development (26). More recently, Sarkar et al. (27) showed that the magnitude of cellular immune responses to E6 or E7 peptides correlates positively with recurrence-free survivals of cervical intraepithelial neoplasia patients following ablative or excisional treatment. It is likely that antitumor efficacy of conventional treatment modalities might be increased dramatically by stimulating E6- or E7-specific cellular immunity in patients with cervical cancer.

Using our well-defined animal tumor model, we evaluated the potential benefits of combination therapy using E7 subunit vaccines plus chemotherapy in terms of tumor cure rates, recurrence rates, and long-term immunity with a special focus on the underlying therapeutic mechanisms.

Methods and Materials

Mice and tumors. Female 4- to 6-week-old C57BL/6 mice were purchased from Daehan Biolink Co. (Chungbuk, Korea). Animals were cared for under the guidelines of Institutional Animal Care and Use Committee-approved protocols. TC-1 tumor cells (a kind gift from T.-C. Wu, Johns Hopkins University, Baltimore, MD) were grown in complete RPMI 1640 (10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin) supplemented with 400 µg/mL G418. TC-1 is an E7-expressing tumorigenic cell line. It was established from primary lung epithelial cells of C57BL/6 mice immortalized with HPV16 E6 and E7 and then transformed with an activated *ras* oncogene (28). The tumor cells were washed twice with PBS and injected into mice.

Chemotherapeutic drugs and their delivery. Tumor tissues were excised from animals with established tumors of a TC-1 tumor cell line origin and then sent to Metabio Co., Ltd. (Seoul, Korea) for three-dimensional chemosensitivity test over various chemotherapeutic drugs of our choice as described previously (29). To evaluate the antitumor effect of different delivery methods of chemotherapeutic drugs *in vivo*, tumor-bearing animals were injected i.p., i.v., or intratumorally with cisplatin (0.5 mg/mL; Ildong Pharmaceuticals Co., Ltd., Seoul, Korea) and 5-fluorouracil (5-FU; 5 mg/mL; Choongwae Pharmaceuticals Co., Ltd., Seoul, Korea) at their indicated doses. However, control animals were injected i.p., i.v., or intratumorally with 100 µL PBS.

E7 subunit vaccines and their delivery. For therapeutic treatments with E7 subunit vaccines, tumor-bearing animals were injected s.c. with 20 µg of recombinant E7 proteins in the presence of 20 µg CpG oligonucleotide in a final volume of 100 µL PBS using a 30-gauge needle (Becton Dickinson, Franklin Lakes, NJ). In particular, either recombinant E7 protein or CpG oligonucleotide was also tested to determine individual effects of E7 subunit vaccine components on antitumor therapeutic synergy when combined with cisplatin. As a control, 100 µL PBS was injected instead. In particular, contralateral injection was made at a site distant to tumor sites two to three times at 1-week interval. In this study, recombinant E7 proteins were prepared as reported previously (11). The unmethylated CpG oligonucleotide designated as 1826 (5'-TCCATGACGTTCCCTGACGTT-3') was purchased from GenoTech, Taejeon, Korea. It was synthesized with a nuclease-resistant phosphorothioate backbone, dissolved in water, and then confirmed to have undetectable endotoxin level.

Tumor response to chemotherapy plus immunotherapy. TC-1 cells (2×10^5) were injected s.c. into the right flank of C57BL/6 mice. When tumors were approximately 5 to 8 mm in mean tumor diameter, animals were injected intratumorally with cisplatin and s.c. with E7 subunit vaccines. For tumor rechallenge studies, 4×10^5 TC-1 cells per mouse were injected s.c. into the left flank of C57BL/6 mice. Mice were monitored twice weekly for tumor growth. Tumor size was measured in millimeter using a caliper and recorded as mean diameter $[(a + b) / 2]$, where *a* is the longest surface length and *b* is the width. Cured animals were denoted as those showing complete response for 120 days posttreatment. Percentage cure rates were calculated as follows: (the number of animals cured for 120 days following the first treatment / the number of animals treated) \times 100. Mice were euthanized when tumor size reached >20 mm in mean diameter.

CTL assay. Splenocytes were stimulated *in vitro* with 1 µg E7 CTL peptides (amino acids 49-57) containing MHC class I epitope (30) per milliliter and 25 units of recombinant interleukin-2 (Becton Dickinson) per milliliter for 5 days. The E7 CTL peptide (RAHYNIVTF) was purchased from Pepton (Taejeon, Korea). CTL assay was done in 96-well round-bottomed plates. Cytolysis was determined by quantitatively measuring lactate dehydrogenase activity as reported previously (31). Splenocytes served as an effector cells and TC-1 cells were used as target cells. CTL assay was done with effector and target cells (1×10^4 per well) mixed at varying E:T cell ratios in a final volume of 200 µL. After a 5-h incubation at 37°C, 50 µL cell supernatant was collected to test the amount of lactate dehydrogenase in the cultured medium according to the manufacturer's protocol (Promega, Madison, WI). After background subtraction, the percentage lysis was calculated as follows: $100 \times [(experimental\ release - effector\ spontaneous\ release - target\ effector\ spontaneous\ release) / (target\ maximum\ release - target\ spontaneous\ release)]$.

Adoptive transfer of CTL effector cells. CTL effector cells were prepared as above. The effector cells were washed thrice in PBS and then 1×10^7 effector cells per mouse in 100 µL PBS were injected i.v. into animals with two masses of tumors located distantly on days 0 and 1 following cisplatin treatment.

In vivo depletion of CD4⁺ and CD8⁺ T cells. Depletion studies were done as described previously (10, 11, 32). Briefly, 100 µL ascites fluids were given i.p. on days -3, 0, and 3 of the second treatment with E7 subunit vaccines.

Histologic analysis. Tumor-bearing animals were sacrificed and tumors were excised. The tumor mass was placed in 4% formalin solution. The fixed tumor mass was bisected longitudinally and embedded in paraffin. The 4- to 5-µm sections of the embedded tumor tissues were made and then stained with H&E. Light microscopy was used to identify the status of necrosis, abnormal cell borders, loss or condensation of the nucleus, eosinophilic cytoplasm, and lymphocyte infiltration or inflammation.

SDS-PAGE and Western blot assay. Tumor tissue was homogenized in lysis buffer [0.1 mol/L Tris-HCl (pH 7.8), 0.5% Triton X-100, protease inhibitor cocktail] and then 60 µg tumor tissue lysates were run on 12% SDS-PAGE. Western blot assay was done as described previously (33). In particular, anti-caspase-3 (both 35 and 17 kDa specific) and anti-β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA) and used as primary antibodies.

Statistical analysis. Statistical analysis was done using the ANOVA. Values between different treatment groups were compared. *P* values <0.05 were considered significant.

Results

Selection of chemotherapeutic drugs and their delivery methods in vitro and in vivo. To select a cytotoxic drug for combination therapy with E7 subunit vaccines, an established TC-1 tumor was excised and the tissues were evaluated over various chemotherapeutic drugs using three-dimensional

chemosensitivity test. As shown in Fig. 1A, among tested drugs (cisplatin, paclitaxel, 5-FU, etoposide, irinotecan, mitomycin C, Adriamycin, and ifosfamide), cisplatin and 5-FU displayed the highest inhibition rates against TC-1 tumor growth *in vitro*. However, this was below the threshold level of 30% inhibition rates, suggesting that TC-1 tumors are relatively chemoresistant. We next compared the abilities of delivery methods of cisplatin versus 5-FU delivered *i.p.* at varying doses to induce antitumor therapeutic efficacy. As shown in Fig. 1B, both doses (50 and 75 mg/kg) of 5-FU delivered *i.p.* made all five tested animals dead within 4 days posttreatment. In contrast, cisplatin treated at a dose of 2.5, 5, and 10 mg/kg inhibited tumor growth insignificantly compared with control group. However, all five tested animals survived at these drug doses, except for one drug dose (10 mg/kg) showing one death out of five tested animals. We next evaluated an intratumoral delivery method. In particular, the intratumoral injection volume was limited to 100 μ L. As shown in Fig. 1C, 5-FU delivered intratumorally at 12.5 and 25 mg/kg made all five tested animals dead at 12 and 9 days posttreatment, respectively. In contrast, cisplatin delivered intratumorally at a dose of 2.5 mg/kg showed a significant level of tumor growth inhibition but no death in all tested animals, suggesting that cisplatin might be tested as a drug in combination with immunotherapy. We further compared the abilities of two delivery methods (intratumorally versus *i.v.*) of cisplatin at 2.5 mg/kg to induce tumor growth inhibition (Fig. 1D). When animals with established tumors of 7 mm in mean diameter were treated intratumorally versus *i.v.* with cisplatin, intratumoral delivery inhibited tumor growth significantly more than *i.v.* delivery. Therefore, we chose the intratumoral delivery method of cisplatin to test our hypothesis.

E7 subunit vaccines improved cure and recurrence rates of tumors after local cisplatin therapy. To investigate whether E7 subunit vaccines might enhance tumor response to local cisplatin therapy, tumor-bearing animals were treated intratumorally with cisplatin and then *s.c.* with E7 subunit vaccines. As shown in Fig. 2, treatment with either cisplatin or E7 subunit vaccines alone resulted in no cure of tumors (0% cure rates) but showed delayed tumor growth for a few to 15 days compared with negative controls (0% cure rates). However, cisplatin plus E7 subunit vaccine-treated animals showed complete cure of tumors for 120 days following the first treatment (100% cure rates), which is a dramatic level of therapeutic synergy. We next tested this combination strategy in animals with tumors of 5 mm in diameter, a smaller tumor in size, as we were interested in seeing long-term antitumor therapeutic effects between cisplatin treatment and cisplatin plus E7 subunit vaccine treatment. As shown in Fig. 3, two of five animals treated with cisplatin alone cured tumors for 120 days posttreatment, giving rise to 40% (2 of 5) cure rates. The remaining three failed to cure tumors, one of which displayed regression of tumors at 26 days posttreatment, but the tumors recurred at 54 days posttreatment. However, all animals treated with cisplatin plus E7 subunit vaccines showed 100% cure rates without any recurring tumors, supporting a dramatic role of E7 subunit vaccines plus cisplatin in improving both tumor cure and recurrence rates. We further tested whether long-term antitumor immunity could be achieved in these surviving animals. When two surviving animals by cisplatin treatment alone and all five surviving animals by treatment with cisplatin plus E7 subunit vaccines were rechallenged with TC-1 cells at 125 days

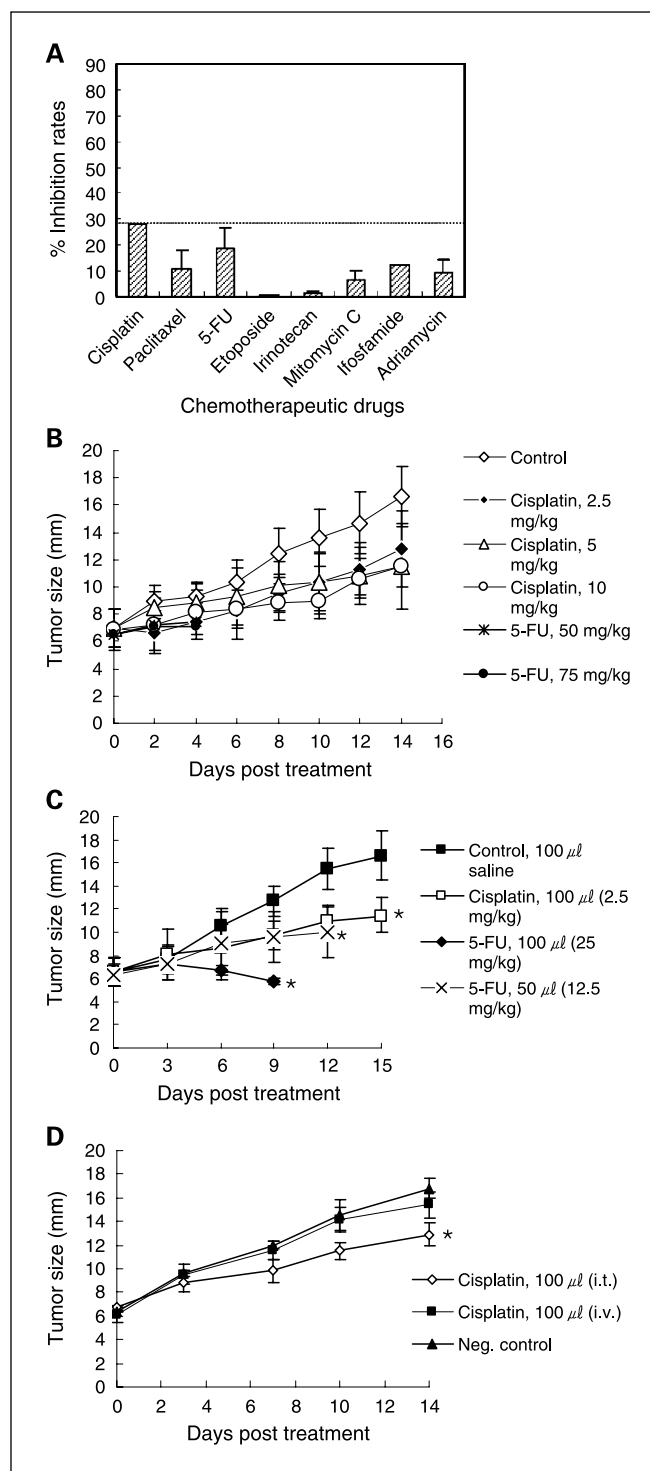


Fig. 1. Selection of chemotherapeutic drugs and their delivery methods *in vitro* and *in vivo*. **A**, a series of chemotherapeutic drugs was selected for three-dimensional chemosensitivity test. Tumor masses were excised from tumor-bearing animals and then sent to Metabio for the test. **B**, two selected drugs, cisplatin and 5-FU, at different doses were delivered *i.p.* to each group of animals ($n = 5$) with established tumors of 7 mm in diameter twice at 1-wk interval. **C**, cisplatin and 5-FU at different doses were delivered intratumorally to each group of animals ($n = 5$) with established tumors of 7 mm in diameter twice at 1-wk interval. **D**, cisplatin at a dose of 2.5 mg/kg (100 μ L) was delivered intratumorally (*i.t.*) versus *i.v.* to each group of animals ($n = 5$) with established tumors of 7 mm in diameter twice at 1-wk interval. Columns and points, mean percentage inhibition rates and mean tumor sizes; bars, SD. This was repeated with similar results. *, statistically significant at $P < 0.05$ using ANOVA compared with control animals.

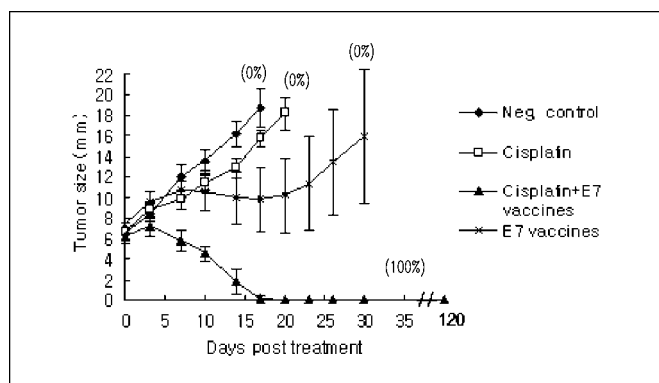


Fig. 2. Antitumor therapeutic effects of cisplatin + E7 subunit vaccines in combination in tumor-bearing animals. Each group of mice ($n = 10$) with established tumors of 7 mm in mean diameter was injected intratumorally with cisplatin (2.5 mg/kg) twice at 1-wk interval and/or s.c. with E7 subunit vaccines thrice at 1-wk interval. Columns, mean tumor sizes; bars, SD. This was repeated with similar results. Number in parenthesis, percentage cure rates of tumor. The percentage cure rates were calculated as follows: (the number of animals cured for 120 d following the first treatment / the number of animals treated) \times 100.

posttreatment, all animals treated with cisplatin alone showed tumor formation. In particular, the tumor growth pattern was exactly the same as that of age-matched naive controls (data not shown). In contrast, animals treated with cisplatin plus E7 subunit vaccines eventually showed complete tumor regression. Overall, 10 of 10 tumor-controlled animals by treatment with E7 subunit vaccines plus cisplatin protected tumor rechallenge, whereas zero of three tumor-controlled animals by treatment with cisplatin alone protected tumor rechallenge (100% versus 0% in protection rates from tumor rechallenge). These data suggest that combination therapy using cisplatin plus E7 subunit vaccines results in inducing long-term antitumor immunity, which seems to be responsible for prevention of any tumor recurrence in these animal groups.

Both components of E7 subunit vaccines were required for induction of CTL as well as antitumor therapeutic synergy when combined with cisplatin. To investigate whether both compo-

nents of E7 subunit vaccines (recombinant E7 proteins or CpG oligonucleotide) are required for displaying therapeutic synergy when combined with cisplatin, tumor-bearing animals were treated intratumorally with cisplatin and s.c. with individual components of E7 subunit vaccines. As shown in Fig. 4A, no therapeutic synergy was observed in tumor-bearing animals under treatment with either cisplatin plus E7 proteins or cisplatin plus CpG oligonucleotide. However, animals treated with cisplatin plus E7 subunit vaccines showed therapeutic synergy that we observed previously, suggesting that therapeutic synergy is achieved when both components of E7 subunit vaccines are used together with cisplatin. We also tested *in vitro* CTL activities in these animals as we reported previously that CD8⁺ CTL is mainly responsible for antitumor immunity in this model (10, 11, 32). As shown in Fig. 4B, a significant level of E7-specific CTL activities were observed only in animals treated with cisplatin plus E7 subunit vaccines but not in those treated with either cisplatin plus E7 proteins or cisplatin plus CpG oligonucleotide, further suggesting that E7-specific CTL can be inducible only when E7 proteins plus CpG oligonucleotide are used together. These data further show that CTL activities are directly associated with antitumor therapeutic synergy mediated by cisplatin plus E7 subunit vaccines in this model.

To further confirm the importance of CD8⁺ CTL in therapeutic synergy, animals were depleted *in vivo* of CD4⁺ or CD8⁺ T cells during chemoimmunotherapy. As shown in Fig. 4C, cisplatin plus E7 subunit vaccine-treated animals with CD8⁺ T cell depletion showed tumor growth in a manner similar to animals treated with cisplatin alone. However, animals treated with cisplatin plus E7 subunit vaccines in the presence of CD4⁺ T-cell depletion displayed tumor suppression in a manner similar to animals treated with cisplatin plus E7 subunit vaccines, confirming that E7-specific CD8⁺ T cells (CTL) but not CD4⁺ T cells are responsible for therapeutic synergy as effector cells.

E7 subunit vaccine-induced tumor cell depopulation, tumor cell density, and lymphocyte infiltration. Histology of tumors from animals treated with cisplatin and/or E7 subunit vaccines was

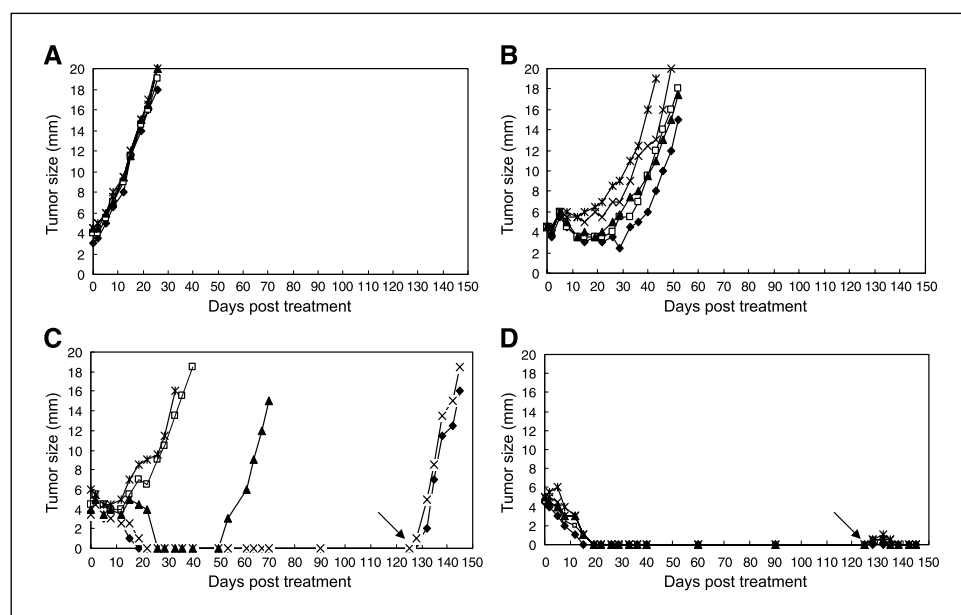


Fig. 3. Long-term antitumor therapeutic effects of cisplatin + E7 subunit vaccines in tumor-bearing animals. Each group of animals ($n = 5$) with established tumors of 5 mm in mean diameter was injected intratumorally with cisplatin (2.5 mg/kg) twice at 1-wk interval and/or s.c. with E7 subunit vaccines thrice at 1-wk interval. At 125 d posttreatment (arrow), two tumor-cured animals by treatment with cisplatin alone and all five tumor-cured animals by treatment with cisplatin + E7 subunit vaccines were rechallenged with 4×10^5 TC-1 cells per mouse. Points, mean tumor sizes of each animal up to 150 d after the first treatment. Nontreatment control (A), E7 subunit vaccines alone (B), cisplatin treatment alone (C), cisplatin + E7 subunit vaccine treatment (D). Arrow, time of tumor rechallenge. This was repeated with similar results. One of the data is shown here.

compared (Fig. 5). Untreated controls were highly cellular and looked squamous carcinoma cell types. Tumors from cisplatin-treated animals also showed a little decreased level of tumor cell density compared with negative control. However, tumors of animals treated with E7 subunit vaccines showed a greater infiltration of lymphocytes and contained large areas of reduced tumor cell density compared with those of control animals. However, the tumors of animals treated with cisplatin plus E7 subunit vaccines were infiltrated heavily with lymphocytes and contained a few tumor cells on the H&E staining.

Comparison of *in vitro* CTL activities versus tumor regression status in animals treated with cisplatin and E7 subunit vaccines. We assumed that apoptotic TC-1 tumor cells by cisplatin treatment might serve as antigen-presenting cells, leading to induction/enhancement of CTL for antitumor therapeutic synergy. To first test whether cisplatin could induce apoptosis in TC-1 tumors *in vivo*, animals were injected intratumorally with cisplatin and then tumor mass was tested for apoptosis. As shown in Fig. 6A, a cleaved form of active caspase-3 (a key mediator of apoptosis) was detected by cisplatin treatment, as opposed to untreated control, showing that local injection of cisplatin can induce apoptosis in TC-1 tumors. We next evaluated the possibility that apoptotic TC-1 tumor cells might serve as antigen-presenting cells for induction/enhancement of Ag-specific CTL *in vivo* and that there might be a direct correlation between *in vitro* CTL activities and tumor regression status in animals under chemoimmunotherapy. To test this, we treated tumor-bearing animals and age-matched naive animals with/without cisplatin and E7 subunit vaccines. Tumor size per animal group was measured at the time of animal sacrifice for *in vitro* CTL assays (Fig. 6B). Figure 6C shows *in vitro* CTL activities in these animals. We observed a similar level of CTL activities in all animal groups treated at least with E7 subunit vaccines. In particular, tumor-bearing animals treated with E7 subunit vaccines alone (tumor size, 11 mm) showed CTL activities similar to those of two other groups [tumor-bearing groups treated with cisplatin plus E7 subunit vaccines (tumor size, 2 mm) and naive groups (no tumor) treated with E7 subunit vaccines alone]. However, a background level of CTL activities were detected in animal groups without immunotherapy [tumor-bearing nontreated groups (tumor size, 20 mm), tumor-bearing groups treated with cisplatin alone (tumor size, 16 mm), and nontreated naive group (no tumor)]. Thus, these data show that there is neither induction nor enhancement of CTL by cisplatin treatment and further that there is no direct correlation between *in vitro* CTL activities and tumor regression status.

Increased sensitivity of cisplatin-treated tumor cells to CTL-mediated killing *in vitro* and *in vivo*. We next hypothesized that cisplatin-treated tumor cells might be more sensitive to CTL-mediated killing. This increased susceptibility might be responsible for antitumor therapeutic synergy. To test this hypothesis, we evaluated tumor cell growth patterns and viability upon treatment *in vitro* with cisplatin at 0.2, 0.5, 1, and 1.5 $\mu\text{g}/\text{mL}$ over a 3-day incubation period. TC-1 cells treated with cisplatin at 0.2, 0.5, and 1 $\mu\text{g}/\text{mL}$ showed a similar level of cell viability to control groups up to a 2-day incubation period (data not shown). However, tumor growth was affected immensely by cisplatin treatment in these groups. In particular, treatment with cisplatin at 1.5 $\mu\text{g}/\text{mL}$ resulted in both decreased

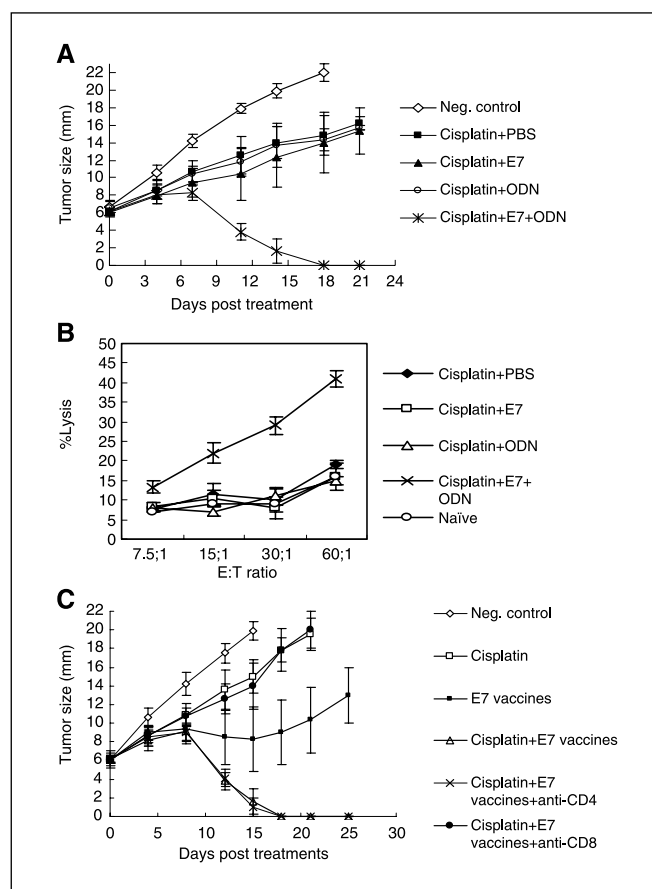


Fig. 4. Antitumor therapeutic effects (A) and *in vitro* CTL activities (B) in tumor-bearing animals by treatment with cisplatin plus individual components of E7 subunit vaccines and antitumor therapeutic effects of *in vivo* depletion of CD8⁺ T cells (C). A, each group of animals ($n = 5$) with established tumors of 6 to 7 mm in mean diameter was treated intratumorally with cisplatin (2.5 mg/kg) twice at 1-wk interval and/or s.c. with PBS, E7 proteins, CpG-oligonucleotide (ODN), or both thrice at 1-wk interval. Tumor size was measured for 21 d following the first treatment. B, animals were sacrificed at 21 d following the first immunization to evaluate *in vitro* CTL activities. C, each group of animals ($n = 5$) with established tumors of 6 to 7 mm in mean diameter was injected intratumorally with cisplatin (2.5 mg/kg) twice at 1-wk interval and s.c. with E7 subunit vaccines thrice at 1-wk interval. At the second treatment, mice were depleted *in vivo* of CD4⁺ or CD8⁺ T cells as described in Materials and Methods. Points, mean percentage lysis and mean tumor sizes; bars, SD. These were repeated with similar results. One of the data is shown here.

tumor cell growth and viability. Therefore, we chose TC-1 tumor cells treated for 2 days with cisplatin at 0.2, 0.5, and 1 $\mu\text{g}/\text{mL}$ as target cells for CTL sensitivity test. As shown in Fig. 6D, CTL activities against TC-1 tumor cells without cisplatin treatment showed 32%, 22%, and 12% at the E:T ratios of 10:1, 5:1, and 2.5:1, respectively. CTL activities against TC-1 tumor cells treated with cisplatin at 0.2 $\mu\text{g}/\text{mL}$ were a bit higher than those against control TC-1 cells without cisplatin treatment. However, CTL activities against TC-1 tumor cells treated with cisplatin at 0.5 $\mu\text{g}/\text{mL}$ showed 67%, 44%, and 22% at the E:T ratios of 10:1, 5:1, and 2.5:1, respectively. This is ~2.0-fold increase in CTL sensitivity by cisplatin treatment. CTL activities against TC-1 tumor cells treated with cisplatin at 1 $\mu\text{g}/\text{mL}$ showed 76%, 53%, and 26% at the E:T ratios of 10:1, 5:1, and 2.5:1, respectively, which is ~2.3-fold increase in CTL sensitivity compared with TC-1 tumor cells without cisplatin treatment. In contrast, MHC-matched control tumor cells

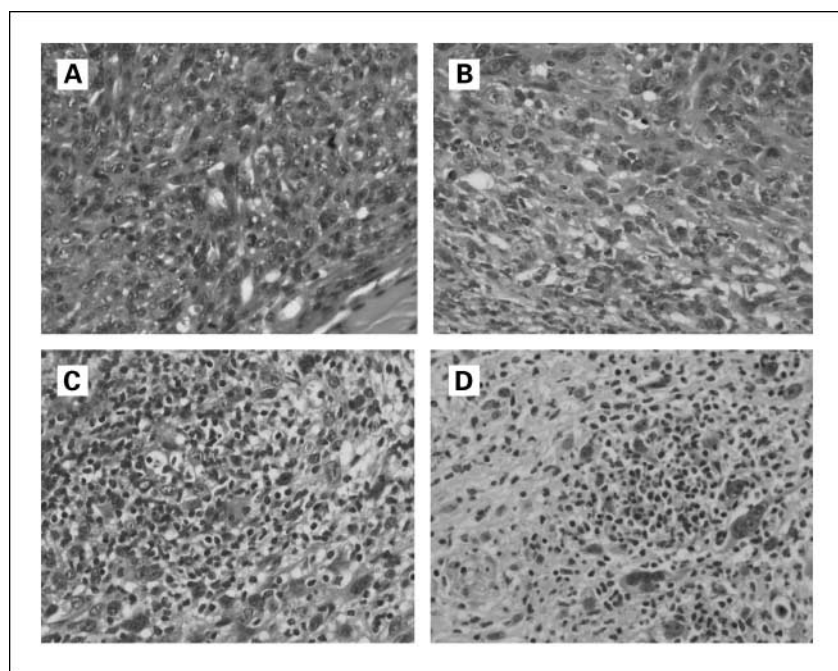


Fig. 5. Histologic evaluation of tumor masses in animals treated by cisplatin and E7 subunit vaccines. Animals with established tumors of 7 mm in mean diameter were injected intratumorally with cisplatin (2.5 mg/kg) twice at 1-wk interval and s.c. with E7 subunit vaccines twice at 1-wk interval. Tumors were excised from animals 2 d after the second treatment and then evaluated by H&E staining. Magnification, $\times 400$. *A*, nontreatment control; *B*, cisplatin treatment alone; *C*, E7 subunit vaccine treatment alone; *D*, cisplatin plus E7 subunit vaccine treatment.

(B16 melanoma cell lines) showed a background level of CTL activity over different E:T ratios. These results suggest that dramatically increased sensitivity of cisplatin-treated tumor cells to CTL-mediated killing might be responsible for antitumor therapeutic synergy. To confirm our *in vitro* findings, we adoptively transferred CTL effector cells to animals bearing two tumor masses located distantly on the abdominal part and then evaluated the regression status of tumors upon local treatment with cisplatin (one tumor site) and PBS (the other tumor site). Figure 6E shows a small difference in tumor size of animals under no adoptive CTL transfer between cisplatin and PBS treatment. However, tumors of animals under adoptive CTL transfer regressed more dramatically by cisplatin treatment, as opposed to PBS treatment (Fig. 6F), suggesting that CTL can remove cisplatin-treated tumors more effectively than nontreated tumors *in vivo*. Thus, these *in vivo* findings match well with our *in vitro* findings and further support that E7 subunit vaccines plus cisplatin-mediated antitumor therapeutic synergy is mediated by increased sensitivity of cisplatin-treated tumors to CTL-mediated killing in this model.

Discussion

As cytotoxic drugs have been used to treat most malignancies, combining these drugs with therapeutic vaccines seems to be increasingly promising in treating the diseases. Our data show a remarkable degree of synergy between cisplatin-based chemotherapy and E7 subunit vaccine-based immunotherapy in treating established E7 expressing TC-1 tumors. In particular, combination effects of cisplatin and E7 subunit vaccines were most prominent by tumor cure rates. For instance, either cisplatin or E7 subunit vaccines alone showed 0% cure rates in animals with tumors of 7 mm in diameter, whereas combined therapy using cisplatin plus E7 subunit vaccines resulted in complete cure of tumors in all animals (100% cure rates). This correlates well with our histologic findings, in which a more

infiltration of lymphocytes as well as a lower level of tumor cell density were detectable in the tumor tissues of animals treated with cisplatin plus E7 subunit vaccines compared with single treatment alone. This is also in line with previous findings that combination of cytotoxic drugs with dendritic cell-based vaccines results in antitumor combination effects in breast cancer and melanoma animal models (22, 23).

The importance of CD8⁺ CTL for eradication of tumors has been reported in a TC-1 tumor challenge model (9–11, 18, 32, 34–37). In our observation, antitumor therapeutic synergy of cisplatin plus E7 subunit vaccines was lost upon depletion of CD8⁺ T cells, but not CD4⁺ T cells, implying that CD8⁺ T cells play an important effector function in antitumor therapeutic synergy. Furthermore, this therapeutic synergy was not observed when animals were treated with cisplatin plus each component of E7 subunit vaccines (E7 proteins and CpG oligonucleotide) compared with cisplatin plus both components of E7 subunit vaccines. Similarly, E7-specific CTL responses were detectable only when animals were treated with cisplatin plus both components of E7 subunit vaccines. These data suggest that Ag-specific CTL activities are directly associated with cisplatin plus E7 subunit vaccine-mediated therapeutic synergy. Furthermore, the tested adjuvant, CpG oligonucleotide has been known to possess immunostimulatory effects (38–40) and induce development of enhanced CTL activity (41, 42).

Recurrent and metastatic tumors after chemotherapy and radiotherapy are often detected in a portion of tumor patients. Therefore, an ideal cancer treatment modality should not only cause tumor eradication but also induce a long-term, systemic antitumor immunity, which is essential for prevention of metastatic and recurrent tumors. In this study, we observed that in the presence of local chemotherapy, E7 subunit vaccines were effective at minimizing recurrence of tumors. For example, for animals with tumors of 5 mm in diameter, three animals controlled tumors at 19, 22, and 26 days posttreatment, but

one last controlled animal displayed recurrence of tumors at 54 days posttreatment. However, such recurrence was not detectable in animals treated with cisplatin plus E7 subunit vaccines. This might be explained by our subsequent findings that long-term antitumor immunity was detectable in tumor-controlled animals by treatment with cisplatin plus E7 subunit vaccines, as opposed to tumor-controlled animals by cisplatin treatment alone. This result suggests that chemotherapy in combination with therapeutic vaccines can mediate prevention of tumor recurrence through induction of long-term antitumor immunity. Furthermore, when tumor cells are metastasized to the other distal sites, the benefits of addition of immunotherapy to chemotherapy might be highlighted as immunotherapy can induce systemic immune responses against metastasized tumor

cells. In this case, however, this benefit might not be expected when the immune system of the host is suppressed by chemotherapy. In animals treated with cisplatin, we observed some decrease in both WBC and absolute lymphocyte counts at 2 days posttreatment but recovery to normal at 5 days posttreatment (data not shown). It seems reasonable that therapeutic vaccination should be initiated during or after immune recovery after chemotherapy. This likely maximizes the synergistic effects of therapeutic vaccines combined with chemotherapy on cure of tumors.

We further evaluated the mechanism(s) as to how antitumor therapeutic synergy was mediated by cisplatin plus E7 subunit vaccines. We first speculated that cisplatin-treated tumor cells might serve as an antigen-presenting cell, leading to

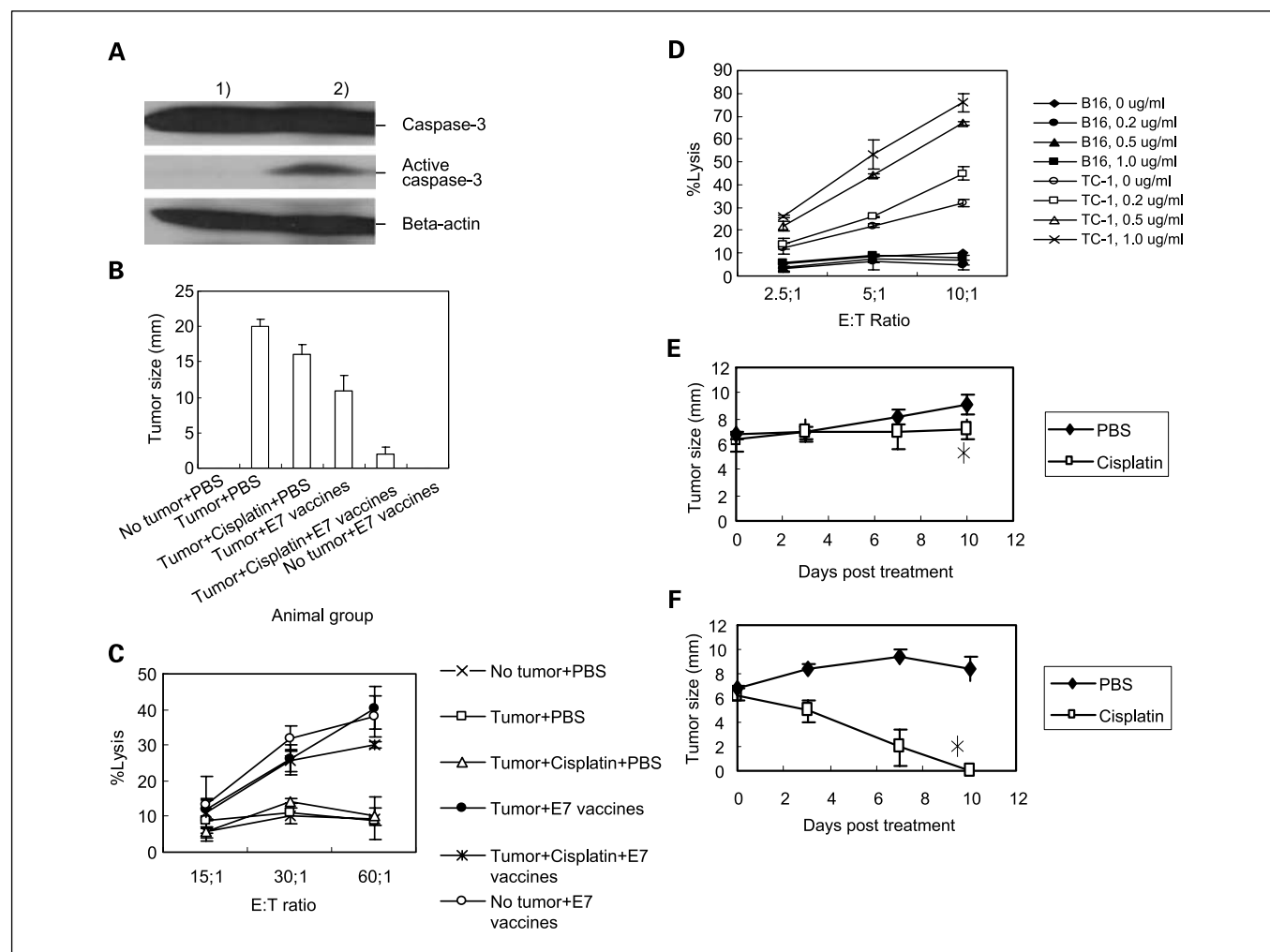


Fig. 6. Detection of apoptosis in TC-1 tumors by local treatment with cisplatin *in vivo* (A), comparison of tumor regression status (B) versus E7-specific CTL activities (C) in animals under chemoimmunotherapy, and increased sensitivity of cisplatin-treated TC-1 tumor cells to CTL-mediated killing *in vitro* (D) and *in vivo* (E and F). A, each group of animals ($n = 2$) with established tumors of 7 to 8 mm in mean diameter was injected intratumorally with cisplatin (2.5 mg/kg) or PBS in a volume of 100 μ L. One day after the injection, tumors were excised and homogenized to obtain tumor tissue lysates for SDS-PAGE and Western blot assay. 1, PBS controls; 2, cisplatin-treated groups. B and C, each group of animals ($n = 5$) with established tumors of 7 to 8 mm in mean diameter or age-matched naive animals was treated intratumorally with cisplatin (2.5 mg/kg) and/or s.c. with E7 subunit vaccines twice at 1-wk interval. At 1 wk following the second injection, the animals were checked for tumor size (B) and then sacrificed to obtain splenocytes for measuring *in vitro* CTL activities (C). D, animals ($n = 10$) were immunized s.c. with E7 subunit vaccines twice at 2-wk intervals. At 3 wk following the second immunization, animals were sacrificed to obtain splenocytes for measuring direct CTL activities. Splenocytes were stimulated *in vitro* for 5 d to obtain effector cells, which were then reacted at different E:T cell ratios with TC-1 tumor cells (target cells). Target cells were treated previously for 2 d with cisplatin at a dose of 0.2, 0.5, and 1 μ g/mL. As a control, a MHC-matched melanoma cell line, B16, was tested as target cells. E and F, each group of animals ($n = 5$) with two tumor masses (6–7 mm) located distantly on the abdominal part was adoptively transferred with either PBS (E) or effector cells (F) prepared as described in (D) followed by local treatment with PBS or cisplatin. Tumor size was measured for 10 d after the first treatment. Columns and points, mean percentage lysis and mean tumor sizes; bars, SD. These were repeated with similar results. *, statistically significant at $P < 0.05$ using ANOVA compared with PBS controls.

enhancement of CTL effector cell activities when subsequently treated with E7 subunit vaccines. This is based on the previous report that cisplatin can cause tumor cell apoptosis (43), which can engage in antigen presentation (44, 45). However, this model of an increase in antigen-presenting capability by TC-1 tumor cell apoptosis does not fit the profile of the induction pattern of *in vitro* CTL. For instance, CTL activities were not detectable in splenocytes of tumor-bearing animals treated with cisplatin alone. Moreover, E7 subunit vaccine-driven CTL activities were not enhanced when combined with cisplatin. We next thought that cisplatin-treated tumor cells could be rendered more susceptible to CTL-mediated killing. This increased sensitivity might be responsible for therapeutic synergy. This hypothesis is supported by our *in vitro* data that cisplatin-treated tumor cells were more sensitive to CTL-mediated killing. For example, tumor cells treated with cisplatin at 0.5 and 1 $\mu\text{g}/\text{mL}$ showed about 2.0- and 2.3-fold increases in CTL sensitivity, respectively, compared with nontreated controls. This *in vitro* data suggests that increased tumor sensitivity to CTL-mediated killing might be the possible mechanisms for therapeutic synergy. This is in line with the previous finding that cytotoxic drugs, such as 5-FU and dacarbazine, can sensitize tumor cells to CTL-mediated apoptosis *in vitro* (46). This hypothesis was further shown by our *in vivo* findings that cisplatin-treated tumor mass regressed more dramatically by adoptive transfer of CTL effector cells, as opposed to nontreated tumor mass. Furthermore, the similar finding was observed when animals with two tumor masses were treated locally with cisplatin versus PBS in the presence of E7 subunit vaccination (data not shown). Therefore, these *in vitro* and *in vivo* data

support the notion that antitumor therapeutic synergy of cisplatin plus E7 subunit vaccines is mediated mainly by increased sensitivity of cisplatin-exposed tumors to CTL-mediated killing.

In conclusion, we observed a dramatic therapeutic synergy between cisplatin and E7 subunit vaccines in animals with established TC-1 tumors, along with long-term antitumor immunity. In particular, both components of E7 subunit vaccines were required for induction of antigen-specific CTL responses as well as therapeutic synergy when combined with local therapy with cisplatin. This therapeutic synergy was abrogated by depletion of CD8⁺ T cells *in vivo*, suggesting an important effector role of CD8⁺ CTL cells for antitumor therapeutic synergy. Moreover, this combined therapy resulted in histologic changes (heavy infiltration of lymphocytes and reduced tumor cell density). Furthermore, increased sensitivity of cisplatin-treated tumors to CTL-mediated killing seemed to be mainly responsible for antitumor therapeutic synergy, as determined by apoptosis, *in vitro* CTL activities, adoptive CTL transfer, and tumor regression. Thus, these data show that E7 subunit vaccines act as a potent enhancer of tumor chemoresponse and that this is mediated by increased sensitivity of cisplatin-treated tumors to CTL-mediated killing. This study further suggests that E7-based therapeutic vaccines have the potential to improve chemotherapy in patients with cervical cancer.

Acknowledgments

We thank Metabio for doing the three-dimensional chemosensitivity test of TC-1 tumors.

References

- zur Hausen H. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* 1991;184:9–13.
- Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989;63:4417–21.
- Scheffner M, Munger K, Bryne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci U S A* 1991;88:5523–7.
- Muderspach L, Wilczynski S, Roman L, et al. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. *Clin Cancer Res* 2000;6:3406–16.
- Zwaveling S, Ferreira Mota SC, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol* 2002;169:350–8.
- Cui Z, Qiu F. Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. *Cancer Immunol Immunother* 2005;54:1180–90.
- Hung CF, He L, Juang J, Lin TJ, Ling M, Wu TC. Improving DNA vaccine potency by linking Marek's disease virus type 1 VP22 to an antigen. *J Virol* 2002;76:2676–82.
- Kim TW, Hung CF, Ling M, et al. Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. *J Clin Invest* 2003;112:109–17.
- Kim TW, Hung CF, Boyd DA, et al. Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6. *Cancer Res* 2004;64:400–5.
- Kim MS, Sin JI. Both antigen optimization and lysosomal targeting are required for enhanced anti-tumor protective immunity in a human papillomavirus E7-expressing animal tumour model. *Immunology* 2005;116:255–66.
- Kim TY, Myoung HJ, Kim JH, et al. Both E7 and CpG-ODN are required for protective immunity against challenge with human papillomavirus 16 (E6/E7)-immortalized tumor cells: involvement of CD4⁺ and CD8⁺ T cells in protection. *Cancer Res* 2002;62:7234–40.
- Cui Z, Huang L. Liposome-polycation-DNA (LPD) particle as a carrier and adjuvant for protein-based vaccines: therapeutic effect against cervical cancer. *Cancer Immunol Immunother* 2005;54:1180–90.
- Preville X, Ladant D, Timmerman B, Leclerc C. Eradication of established tumors by vaccination with recombinant *Bordetella pertussis* adenylate cyclase carrying the human papillomavirus 16 E7 oncoprotein. *Cancer Res* 2005;65:641–9.
- De Bruijn ML, Schuurhuis DH, Vierboom MP, et al. Immunization with human papillomavirus type 16 (HPV16) oncoprotein-loaded dendritic cells as well as protein in adjuvant induces MHC class I-restricted protection to HPV16-induced tumor cells. *Cancer Res* 1998;58:724–31.
- Santin AD, Bellone S, Gokden M, Cannon MJ, Parham GP. Vaccination with HPV-18 E7-pulsed dendritic cells in a patient with metastatic cervical cancer. *N Engl J Med* 2002;346:1752–3.
- Ferrara A, Nonn M, Sehr P, et al. Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. *J Cancer Res Clin Oncol* 2003;129:521–30.
- Kim TG, Kim CH, Won EH, et al. CpG-ODN-stimulated dendritic cells act as a potent adjuvant for E7 protein delivery to induce antigen-specific anti-tumor immunity in a HPV16 (E6/E7)-associated tumor animal model. *Immunology* 2004;112:117–25.
- Lamikanra A, Pan ZK, Isaacs SN, Wu TC, Paterson Y. Regression of established human papillomavirus type 16 (HPV-16) immortalized tumors *in vivo* by vaccinia viruses expressing different forms of HPV-16 E7 correlates with enhanced CD8(+) T-cell responses that home to the tumor site. *J Virol* 2001;75:9654–64.
- Kaufmann AM, Stern PL, Rankin EM, et al. Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 E6 and E7 genes, in women with progressive cervical cancer. *Clin Cancer Res* 2002;8:3676–85.
- Bermudez-Humaran LG, Cortes-Perez NG, Lefevre F, et al. A novel mucosal vaccine based on live *Lactococci* expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. *J Immunol* 2005;175:7297–302.
- Sin JI, Hong SH, Park YJ, Park JB, Choi YS, Kim MS. Antitumor therapeutic effects of E7 subunit and DNA vaccines in an animal cervical cancer model: antitumor efficacy of E7 therapeutic vaccines is dependent on tumor sizes, vaccine doses, and vaccine delivery routes. *DNA Cell Biol* 2006;25:277–86.
- Tong Y, Song W, Crystal RG. Combined intratumoral injection of bone marrow-derived dendritic cells and systemic chemotherapy to treat pre-existing murine tumors. *Cancer Res* 2001;61:7530–5.
- Yu B, Kusmartsev S, Cheng F, et al. Effective combination of chemotherapy and dendritic cell administration for the treatment of advanced-stage experimental breast cancer. *Clin Cancer Res* 2003;9:285–94.
- Green JA, Kirwan JM, Tierney JF, et al. Survival and recurrence after concomitant chemotherapy and radiotherapy for cancer of the uterine cervix: a

- systematic review and meta-analysis. *Lancet* 2001;358:781–6.
25. Nakagawa M, Stites DP, Farhat S, et al. Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia. *J Infect Dis* 1997;175:927–31.
 26. Nakagawa M, Stites DP, Patel S, et al. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. *J Infect Dis* 2000;182:595–8.
 27. Sarkar AK, Tortolero-Luna G, Follen M, Sastry KJ. Inverse correlation of cellular immune responses specific to synthetic peptides from the E6 and E7 oncoproteins of HPV-16 with recurrence of cervical intraepithelial neoplasia in a cross-sectional study. *Gynecol Oncol* 2005;99:S251–61.
 28. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;56:21–6.
 29. Furukawa T, Kubota T, Hoffman RM. Clinical applications of the histoculture drug response assay. *Clin Cancer Res* 1995;1:305–11.
 30. Feltkamp MC, Smits HL, Vierboom MP, et al. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* 1993;23:2242–9.
 31. Corr M, von Damm A, Lee DJ, Tighe H. *In vivo* priming by DNA injection occurs predominantly by antigen transfer. *J Immunol* 1999;163:4721–7.
 32. Ahn WS, Bae SM, Kim TY, et al. A therapy modality using recombinant IL-12 adenovirus plus E7 protein in a human papillomavirus 16 E6/E7-associated cervical cancer animal model. *Hum Gene Ther* 2003;14:1389–99.
 33. Sin JI, Sung JH, Suh YS, Lee AH, Chung JH, Sung YC. Protective immunity against heterologous challenge with encephalomyocarditis virus by VP1 DNA vaccination: effect of co-injection with a granulocyte, macrophage-colony stimulating factor gene. *Vaccine* 1997;15:1827–33.
 34. Liu DW, Tsao YP, Kung JT, et al. Recombinant adeno-associated virus expressing human papillomavirus type 16 E7 peptide DNA fused with heat shock protein DNA as a potential vaccine for cervical cancer. *J Virol* 2000;74:2888–94.
 35. Hung CF, Cheng WF, Hsu KF, et al. Cancer immunotherapy using a DNA vaccine encoding the translocation domain of a bacterial toxin linked to a tumor antigen. *Cancer Res* 2001;61:3698–703.
 36. Cheng WF, Hung CF, Hsu KF, et al. Cancer immunotherapy using Sindbis virus replicon particles encoding a VP22-antigen fusion. *Hum Gene Ther* 2002;13:553–68.
 37. Kim TW, Hung CF, Kim JW, et al. Vaccination with a DNA vaccine encoding herpes simplex virus type 1 VP22 linked to antigen generates long-term antigen-specific CD8-positive memory T cells and protective immunity. *Hum Gene Ther* 2004;15:167–77.
 38. Weiner GJ, Liu H-M, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc Natl Acad Sci U S A* 1997;94:10833–7.
 39. Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J Immunol* 1998;160:870–6.
 40. Scott Gallichan W, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. *J Immunol* 2001;166:3451–7.
 41. Krieg AM, Yi A-K, Matson S, et al. CpG motifs in bacterial DNA trigger direct B cell activation. *Nature* 1995;374:546–9.
 42. Warren TL, Bhatia SK, Acosta AM, et al. APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J Immunol* 2000;165:6244–51.
 43. Kim JH, Ajaz M, Lokshin A, Lee YJ. Role of antiapoptotic proteins in tumor necrosis factor-related apoptosis-inducing ligand and cisplatin-augmented apoptosis. *Clin Cancer Res* 2003;9:3134–41.
 44. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res* 1999;59:734–41.
 45. Zisman A, Ng CP, Pantuck AJ, Bonavida B, Beldegrun AS. Actinomycin D and gemcitabine synergistically sensitize androgen-independent prostate cancer cells to Apo2L/TRAIL-mediated apoptosis. *J Immunother* 2001;24:459–71.
 46. Yang S, Haluska FG. Treatment of melanoma with 5-fluorouracil or dacarbazine *in vitro* sensitizes cells to antigen-specific CTL lysis through perforin/granzyme- and Fas-mediated pathways. *J Immunol* 2004;172:4599–608.

Clinical Cancer Research

Therapeutic Synergy of Human Papillomavirus E7 Subunit Vaccines plus Cisplatin in an Animal Tumor Model: Causal Involvement of Increased Sensitivity of Cisplatin-Treated Tumors to CTL-Mediated Killing in Therapeutic Synergy

Sung Hwa Bae, Young-Ja Park, Jae-Bok Park, et al.

Clin Cancer Res 2007;13:341-349.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/13/1/341>

Cited articles This article cites 45 articles, 26 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/13/1/341.full#ref-list-1>

Citing articles This article has been cited by 4 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/13/1/341.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://clincancerres.aacrjournals.org/content/13/1/341>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.