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A chitosan hydrogel-based cancer drug delivery system exhibits synergistic antitumor effects by combining with a vaccinia viral vaccine

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Abstract

Cancer treatment combining chemotherapy and immunotherapy has been vigorously exploited to further improve cancer therapeutic efficacy. This study investigated a new chemoimmunotherapy approach utilizing hydrogel as a local anti-cancer drug delivery system. Chitosan hydrogel containing doxorubicin (CH-DOX) and vaccinia virus vaccine expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1) were used as chemoimmunotherapeutic agents. It was found that intratumoral injection of CH-DOX effectively inhibited tumor growth itself and, in addition, exhibited a synergistic antitumor effect in combination with a vaccinia virus-based vaccine. This combination did not decrease but rather increased the number of tumor-specific $CD8^+$ T cells primed by vaccinia virus-mediated vaccination; the resulting antitumor effects were further improved up to 60 days as compared with monotherapy after tumor challenge, and the survival of tumor-bearing mice was dramatically prolonged. This study is a pioneer report that demonstrates the use of a biodegradable hydrogel system as an anti-cancer drug delivery system for successful chemoimmunotherapy. It is hoped that, this study can provide a foundation for a rational approach to improve antitumor efficacy of chemoimmunotherapy.

Keywords: Hydrogel; Doxorubicin; Vaccinia vaccine; Chemoimmunotherapy

1. Introduction

Many clinical or preclinical trials for cancer treatment have developed multi-modality treatment regimens that can be more effective than the single use of a cancer therapeutic agent (Lake and Robinson, 2005; Nowak et al., 2003). However, it has been widely assumed that the use of combined therapy of chemotherapy and immunotherapy for cancer treatment is unrelated to enhanced synergistic antitumor effects due to immune tolerance and suppression induced by the immunotoxicity of the anti-cancer drugs.

The therapeutic efficacy of chemoimmunotherapy for cancer has often been limited by the serious side effect of the toxicity to healthy tissue of the cancer drugs. To overcome this limitation, local treatment of tumors using varied cancer therapy strategies, such as radiotherapy (Barcellos-Hoff et al., 2005), the use of anti-angiogenesis agents (Bocci et al., 2002), prodrug strategies (Denny, 2004), or the use of hydrogel-based drug delivery systems (Konishi et al., 2003), have been utilized as a means to make the therapy more effective by enhancing the efficacy of the local toxic agent against tumors with minimal damage to the host immune systems. One of the approaches to administer cytotoxic-chemotherapeutic agents, the use of hydrogel systems

Abbreviations: CH-DOX, chitosan hydrogel containing doxorubicin; Sig/E7/LAMP-1, sorting signal of the lysosome-associated membrane protein type-1; Vac-Sig/E7/LAMP-1, vaccinia vaccine expressing Sig/E7/LAMP-1; Vac-WT, wild type vaccinia virus; IFN-γ, interferon-γ; APC, antigen presenting cell; MHC, major histocompatibility complex; HPV 16, human papilloma virus 16; CTL, cytotoxic T lymphocyte; EGCG, epigallocatechin gallate

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for intratumoral injection, has been developed to reduce inherent toxicity of anti-cancer drugs (Olson et al., 2003). Especially, the use of injectable thermosensitive hydrogel systems for local drug delivery to clinically treat cancer has been developed as a delivery system for the administration of anti-cancer drugs (Han et al., 2004; Chenite et al., 2000; Jeong et al., 1997; De Groot et al., 2002).

We have focused on the further development of novel hydrogel systems using chitosan as a polymer matrix due to its biocompatibility and biodegradability, and have demonstrated previously that the chitosan solution displayed a sol–gel phase transition at physiological pH and in a temperature-dependent manner and formed an endothermic hydrogel after subcutaneous injection into mice. Moreover, we have confirmed *in vivo* biodegradation of chitosan hydrogel in a mouse model after subcutaneous injection (Han et al., 2004). Thus, the chitosan hydrogel system could be a promising thermosensitive injectable delivery system to administer local anti-cancer drugs with a reduction of the severe side effects of the drugs.

Most tumors express an array of antigens that could act as targets for immune-mediated destruction, and a number of potential immunotherapies have emerged to exploit this property (Nowak et al., 2002; Melief et al., 2000). Notably, to enhance the immunity for tumors, various vaccine systems have been developed to elicit T cell-mediated immunotherapy for the treatment of tumors without side effects and provide long-term treatment efficacy (Kim et al., 2003, 2004). Among them, the use of vaccinia virus vaccines have become one of the most prevalent vectors for antigen-specific immunotherapy and show promise (Hsieh et al., 2004). Vaccinia vectors have elicited potent antigen-specific, T cell-mediated immune response, and have generated antitumor effects in numerous preclinical models (Ji et al., 1998). We have developed vaccinia virus systems against cervical cancer, which is highly associated with human papilloma virus (HPV) infection. To overcome the weak antigenicity of E7, wild type E7 was linked with the sorting signal of the lysosome-associated membrane protein 1 (Sig/LAMP-1), leading to the enhancement of MHC class I and class II presentation of the encoded E7 antigen by dendritic cells (DCs) to T cells. Vaccinia virus expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1) generated greater antitumor immunity against an E7 expressing murine tumor model, TC-1, than Vac-E7 (Kim et al., 2003; Hsieh et al., 2004).

Though immunotherapies including vaccinia virus-mediated treatments are generally effective at least against small tumors in murine tumor models, the therapeutic efficacy of them against established tumors was not satisfied (Lin et al., 2003). One of possible reasons might be due to the fast growth of the tumors. Most cytotoxic cancer drugs including DOX generate a quick antitumor effect to fast-growing tumor cells. Furthermore, novel thermosensitive chitosan hydrogel system we have developed allows us to treat tumors locally without the severe side toxicity of the cancer drugs and damaging the immune system by direct intratumoral injection. From these reasons, we hypothesized that the combination of chemotherapy using chitosan hydrogel containing DOX (CH-DOX) with immunotherapy using Vac-Sig/E7/LAMP-1 might act synergistically against fast-growing tumors. In addition, the combined treatment could potentially exploit the adjuvant effects of enhanced immune response by enhancing the presentation of tumor-specific antigens to the immune system, called 'cross-presentation' (Zagozdzon et al., 1988; Van der Most et al., 2006).

In this study, we investigated a synergistic antitumor immunity induced by the combination of Vac-Sig/E7/LAMP-1 and CH-DOX. Interestingly, we observed a significant increase of E7-specific $CD8^+$ T cell immunity after combining Vac-Sig/E7/LAMP-1 vaccination with intratumoral injection of CH-DOX. The possible mechanisms of interaction will be discussed.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice (5–6 weeks old and 20 g) were purchased from Daehan Biolink (Chungbuk, Korea). All of the procedures for animal experimentation were performed according to approved protocols and in accordance with recommendations of the NIH guideline for the proper use and care of laboratory animals.

2.2. Cell line

The HPV-16 E7-expressing murine tumor model, TC-1, has been described previously (Olson et al., 2003; Jeong et al., 1997). In brief, HPV-16 E6, E7, and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells to generate TC-1. The cells were grown in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum (FBS), 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids, and 0.4 mg/ml G418 at 37 °C in a 5% CO_2 incubator. Cells were maintained within their exponential growth phase.

2.3. Preparation of CH-DOX

CH-DOX was used as a chemotherapeutic agent. We have previously described the preparation, composition, thermosensitivity, biocompatibility, and biodegradability of chitosan hydrogel system as an *in vitro* or *in vivo* depot system (Han et al., 2004). Briefly, chitosan solution (medium molecular weight of 161 kDa, viscosity of 200,000 cps and a degree of deacetylation of 80%) was obtained by dissolving 400 mg of chitosan in 18 ml of 0.1 M HCl solution. Glycerol 2-phosphate disodium salt hydrate (β-GP) solution was prepared by dissolving 2 g of β-GP in 1 ml of distilled water. The chitosan solution was cooled to 4 °C and continuously stirred while adding 1 ml of β-GP solution and the final volume was brought to 20 ml with the addition of distilled water. The final concentration of chitosan in the mixture is 2% (w/v). It was successfully formed at body temperature and physiological pH *in vivo* after subcutaneous injection into mice. In addition, chitosan hydrogel showed an *in vivo* degradation after subcutaneous injection into mice. Notably, the mice did not demonstrate severe side effects such as pus and inflam-

mation and maintained a healthy appearance after implantation of the hydrogel.

2.4. Vaccinia virus vaccine

The preparation of vaccinia virus vaccine encoding E7 has also been described previously (Lin et al., 1996, 2003; Hsieh et al., 2004). Wild type vaccinia virus (Vac-W.T.) and vaccinia virus expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1) were amplified by infecting TC-1 cells *in vitro* according to a standard protocol. The viral stocks were preserved at −70 °C prior to vaccination. Before use, the virus was thawed at 37 °C. The liquid phase stock was centrifuged at 1500 rpm for 2 min. After centrifugation, the stocks were diluted with minimal essential medium (MEM) without FBS to a final concentration of 3×10^8 plaque-forming units (PFU)/ml. Mice were immunized intraperitoneally with 3×10^6 PFU of the vaccinia vaccines (0.1 ml of the diluted vaccine).

2.5. Antitumor activity

5×10^5 cells of murine TC-1 cervical cancer in 20 μl were carefully inoculated into C57BL/6 mice subcutaneously. After designed days following tumor inoculation, therapeutic agents were injected intratumorally or intravenously at a dose of 6 mg DOX/kg body weight. For the determination of tumor volume, each individual tumor size was measured with a caliper and the tumor volume was calculated using the following equation:

$$\text{tumor volume}\,(\text{mm}^3) = \frac{\text{width} \times \text{length}^2}{2} \quad (1)$$

2.6. Flow cytometry analysis

Cell surface marker staining of CD8 and intracellular cytokine staining for interferon-γ (IFN-γ), as well as flow cytometry analysis were performed as described previously (Kim et al., 2003, 2004; Hsieh et al., 2004). Briefly, splenocytes were harvested from mice after chemoimmunotherapy. Before intracellular cytokine staining, 5×10^6 pooled splenocytes from each group were incubated for 16 h with 1 μg/ml (RAHYNIVTF) peptide containing an MHC class I epitope (aa 49–57) for detecting E7-specific $CD8^+$ T cell precursors. Intracellular IFN-γ staining and flow cytometry analysis were performed. Analysis was performed on a Becton-Dickinson FACScan with CellQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA, USA).

2.7. Statistical analysis

All of the data expressed as means standard deviation (S.D.) are representative of at least three different experiments. Data for the intracellular cytokine staining with flow cytometry analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using a Student's *t*-test. All *p*-values <0.05 were considered significant.

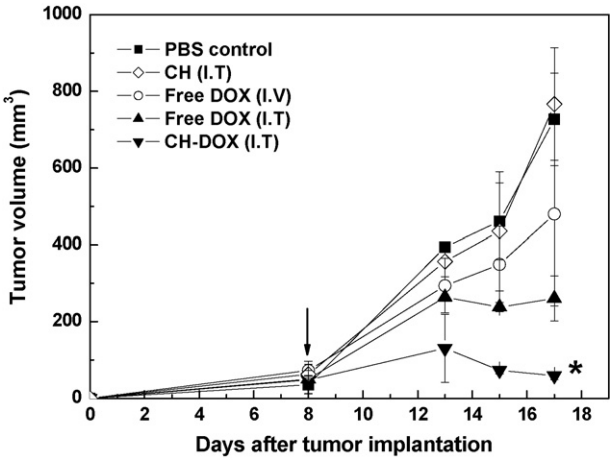

Fig. 1. Tumor growth inhibition in tumor-bearing mice with different injection route at a dose of 6 mg DOX/kg body weight. 5×10^5 TC-1 cells were inoculated into mice subcutaneously. The arrow indicates the injection day of the chemotherapeutic agent after TC-1 cell inoculation (*$p < 0.005$). The data presented are from one representative experiment of the three performed, and represent the mean of six mice ± S.D.

3. Results

3.1. Intratumoral CH-DOX injection enhances antitumor activity

The antitumor activity of free DOX and CH-DOX was evaluated after intravenous or intratumoral injection in TC-1 tumor-bearing C57BL/6 mice (six per group) at a dose of 6 mg DOX/kg body weight, and the results were shown in Fig. 1. CH-DOX as a chemotherapeutic agent was successfully formed as a hydrogel after intratumoral injection as described previously (Han et al., 2004). Intratumoral injection of chitosan hydrogel (CH) alone failed to inhibit tumor growth when compared to PBS, confirming that the hydrogel itself does not have any therapeutic effect against tumor cells. In contrast, injection of free DOX decreased the tumor growth significantly after direct intratumoral injection as compared to intravenous injection. The intratumoral injection of CH-DOX, however, resulted in enhanced antitumor activity than that of free DOX (*$p < 0.005$). This greater effectiveness of the intratumoral injection of CH-DOX is presumably due to the local release of the drug into the tumor site directly and the prolonged exposure of tumor cells to the drug as compared with systemic administration of free DOX (Konishi et al., 2003). Thus, the use of intratumoral injected CH-DOX was selected for further study.

3.2. Determination of Vac-Sig/E7/LAMP-1 vaccination time contributes to enhance antitumor effect and immune response

To optimize the treatment schedule, we immunized the TC-1 tumor-bearing mice with Vac-Sig/E7/LAMP-1 and further treated with CH-DOX at various times as described in Fig. 2b. To evaluate the antitumor effects of treatments, tumor volume was measured at 17 days after tumor inoculation. As shown

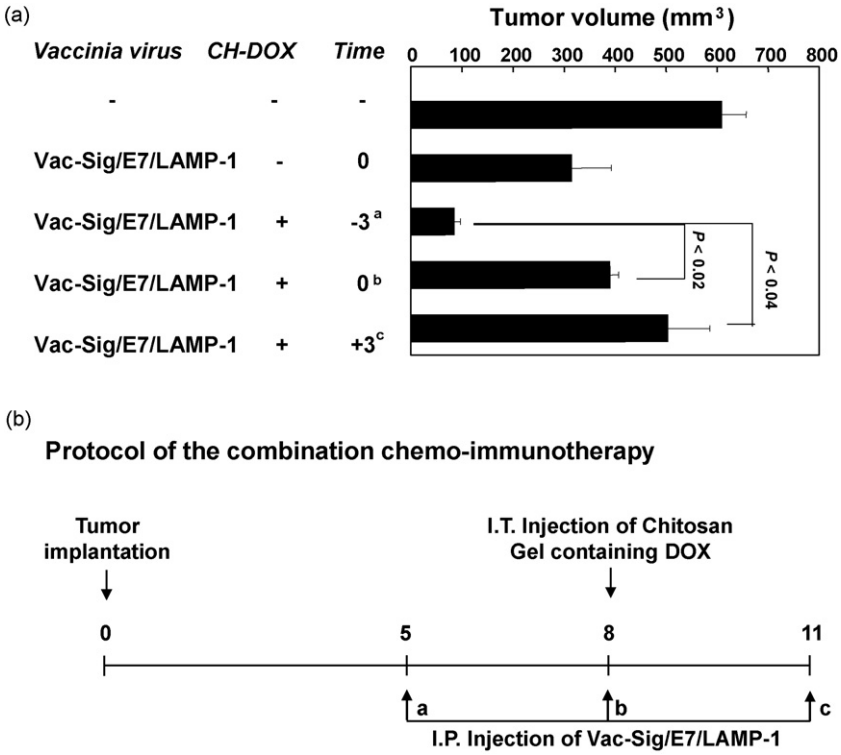

Fig. 2. Determination of the optimum vaccination time of Vac-Sig/E7/LAMP-1 in combination with CH-DOX. The antitumor effect of combined therapy with different Vac-Sig/E7/LAMP-1 vaccination times (a). Experimental protocol of combined therapy (b). TC-1 bearing mice with Vac-Sig/E7/LAMP-1 were immunized at 0 or 3 days before or after tumor treatment with CH-DOX at a dose of 6 mg DOX/kg. The data presented are from one representative experiment of the two performed, and represent the mean of three mice ± S.D.

in Fig. 2a, vaccination at 3 days before tumor treatment with CH-DOX generated the smallest tumor volume. In contrast, vaccination of TC-1 bearing mice with Vac-Sig/E7/LAMP-1 at 0 or 3 days after treatment with CH-DOX failed to decrease tumor volume. Thus, 3 days before CH-DOX intratumoral injection was selected for an optimized vaccination time.

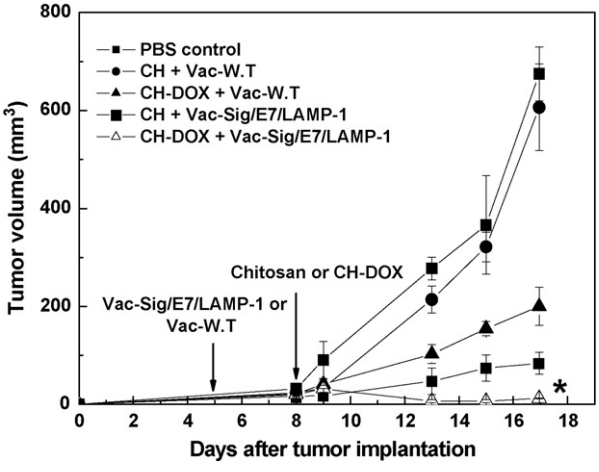

Fig. 3. Tumor growth inhibition by the combination of CH-DOX and Vac-Sig/E7/LAMP-1 in TC-1 bearing mice. The experimental protocol was shown in Fig. 2(b). Vac-Sig/E7/LAMP-1 was vaccinated at 3 days before CH-DOX treatment. The arrow indicates the injection day of chemotherapeutic or immunotherapeutic agents after TC-1 cell implantation into mice. The antitumor activity of combined therapy of CH-DOX and Vac-Sig/E7/LAMP-1 was monitored for 17 days (*$p < 0.01$). The data presented are from one representative experiment of the two performed, and represent the mean of six mice ± S.D.

3.3. Combined therapy using a hydrogel and Vac-Sig/E7/LAMP-1 generate better antitumor effects than monotherapy

To evaluate the increasing synergistic antitumor effects of the combined therapy, we performed an *in vivo* tumor treatment experiment on the basis of the protocol selected in Fig. 2. Empty chitosan hydrogel and Vac-W.T. were used as negative controls. The tumor volume was monitored for 17 days after tumor inoculation and visual images of tumors produced in mice at 17 days were captured. As shown in Fig. 3, the combination of CH-DOX and Vac-Sig/E7/LAMP-1 led to the highest tumor suppression, although there are significant antitumor effects in the group treated with empty CH plus Vac-Sig/E7/LAMP-1 or CH-DOX plus Vac-W.T (*$p < 0.01$). In contrast, all mice co-treated with empty hydrogel and Vac-W.T. had a similar tumor volumes as compared to mice treated solely with PBS.

3.4. Combination of CH-DOX treatment with Vac-Sig/E7/LAMP-1 enhances E7-specific $CD8^+$ T cell immune response

We assessed the antitumor immune response in the mice treated as described in Fig. 3 by performing flow cyto-

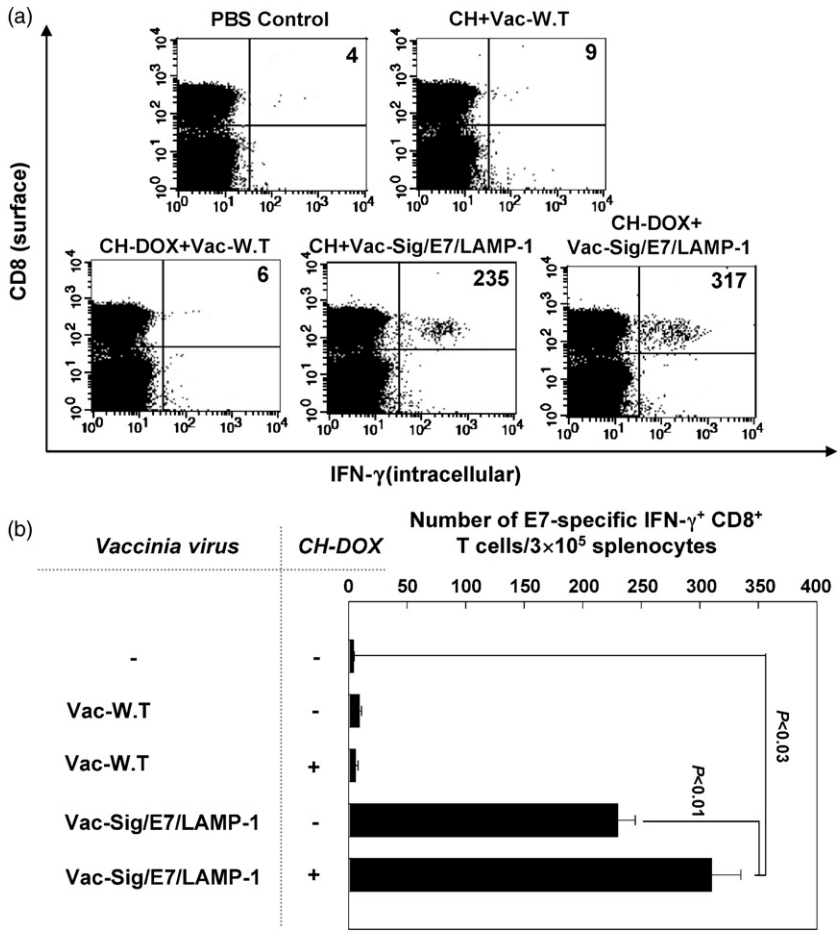

Fig. 4. Flow cytometry analysis of E7-specific IFN-γ-secreting $CD8^+$ T cells in mice treated with the combination of CH-DOX and Vac-Sig/E7/LAMP-1. As described in Fig. 3, the antitumor effect of combined therapy was the highest as compared to other monotherapies. The immune response at above mentioned time (as shown in Fig. 3(a), the result at 17 days) was confirmed. Cell surface marker staining of CD8 and intracellular cytokine staining for interferon-γ (IFN-γ), as well as flow cytometry analysis were performed. Mice (six per group) were sacrificed at 17 days and then splenocytes were harvested. Before intracellular cytokine staining, 5×10^6 pooled splenocytes from each group were incubated for 16 h with 1 μg/ml (RAHYNIVTF) peptide containing an MHC class I epitope (aa 49–57) for detecting E7-specific $CD8^+$ T cell precursors. Immune response of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors/3×10^5 spenocytes for combined therapy was more increased than following the other monotherapeutic strategy (a). Bar graph depicting the number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors/3×10^5 splenocytes (b). The data presented in this figure are from one representative experiment of the three performed, and represent the mean of three mice ± S.D.

metric analysis by determining the number of E7-specific IFN-γ-secreting $CD8^+$ T cells. As shown in Fig. 4a and b, the combination of Vac-Sig/E7/LAMP-1 with CH-DOX treatment resulted in a remarkable increase in the number of E7-specific IFN-γ-secreting $CD8^+$ T cells compared with that of CH- or CH-DOX plus wild type vaccine controls or PBS alone. Notably, the combined therapy of Vac-Sig/E7/LAMP-1 with CH-DOX generated significantly more E7-specific IFN-γ-secreting $CD8^+$ T cells than that of Vac-Sig/E7/LAMP-1 vaccine with an empty chitosan hydrogel. This enhancement of E7-specific $CD8^+$ T cell immune response might not be caused by an adjuvant effect of CH-DOX since the combined therapy of CH-DOX with Vac-WT failed to generate a significant E7-specific $CD8^+$ T cell immune response. Thus, our results indicate that CH-DOX does not hamper but rather enhances the tumor-specific $CD8^+$ T cell immune response induced by Vac-Sig/E7/LAMP-1.

3.5. Combined therapy of CH-DOX with Vac-Sig/E7/LAMP-1 vaccinia virus vaccine increases long-term antitumor activity and mice survival than monotherapy alone

In the treatments of tumors, the efficacy of antitumor effects largely depends on their durability and sustenance. In that point of view, the long-term therapeutic effects were assessed in terms of tumor volume (Fig. 5a) and survival (Fig. 5b) of mice treated as described in Fig. 3. As shown in Fig. 5a, co-treatment with Vac-Sig/E7/LAMP-1 and CH-DOX exhibited a potent suppression of tumor growth at least up to 60 days after tumor challenge. In the case of long-term survival of the mice, as shown in Fig. 5b, the combination of CH-DOX and Vac-Sig/E7/LAMP-1 was more effective than any single therapy at least up to 70 days after tumor challenge. Notably, severe side effects such as weight loss caused by DOX were not observed in groups of the mice treated with CH-DOX in com-

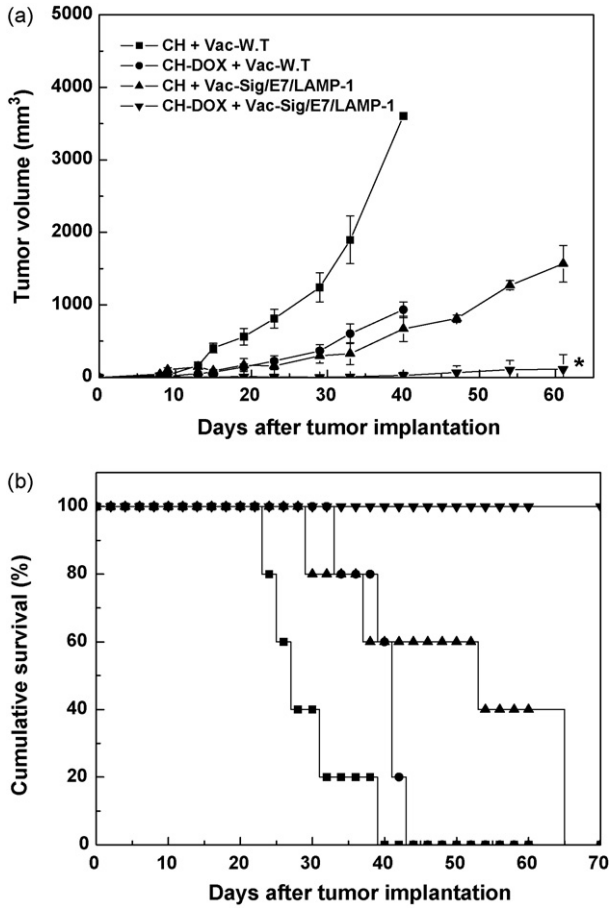

Fig. 5. Long-term tumor growth inhibition of combined therapy in tumor-bearing mice. 5×10^5 murine TC-1 cells were inoculated into the mice subcutaneously and the animals were treated with a combination of Vac-Sig/E7/LAMP-1 and CH-DOX (6 mg/kg), following protocol presented in Fig. 2(b). Mice were vaccinated with Vac-Sig/E7/LAMP-1 at 3 days before CH-DOX treatment. Antitumor activity of long-term tumor growth inhibition was monitored for 60 days by measuring tumor volume of live mice ($^*p < 0.005$) (a). Cumulative survival of mice by combination of CH-DOX and Vac-Sig/E7/LAMP-1 (b). Data of (a) and (b) are from an independent and representative experiment of the two performed, and represent the mean of five mice ± S.D.

bination of Vac-W.T. or Vac-Sig/E7/LAMP-1 to the end of this experiment.

4. Discussion

Immunotherapy and chemotherapy are generally effective against small-sized tumors, but many tumors grow rapidly and, eventually, fast-growing tumors become resistant to both therapies as seen in both preclinical and clinical cases. Therefore, to overcome this intrinsic resistance of the established tumor, effective cancer therapeutic strategies have been developed (Lake and Robinson, 2005; Van der Most et al., 2006). Among them, multi-modality treatment regimens which combine immunotherapy with apoptosis-induced chemotherapeutic drugs have been widely studied in various clinical or preclinical settings (Lake and Robinson, 2005; Nowak et al., 2003). It has been assumed that combining chemotherapy and immunotherapy in cancer treatment do not result in a synergistic increase of antitumor effects because of immune suppression caused by the immunotoxicity of the cancer drugs (Lake and Van der Most, 2006; Nowak et al., 2006).

Hydrogel systems can be used as a delivery system after administration into a mouse body or tumor tissue directly, which can reduce the systemic side effects of anti-cancer drugs (Han et al., 2004; Chenite et al., 2000; De Groot et al., 2002). Biocompatibility and biodegradability of these systems are key parameters for their medical and pharmaceutical applications. To satisfy these specific properties, we prepared a thermosensitive chitosan hydrogel containing a chemotherapeutic agent, DOX (CH-DOX), as an intratumoral composition for local delivery of an anti-cancer drug. In our previous study, we have reported *in vivo* biodegradation of chitosan hydrogel in a mouse model. The chitosan hydrogel systems used in this study was completely degraded and disappeared for 7–10 days after injection under our experimental conditions (Han et al., 2004). It is notable, however, that the release speed of DOX from chitosan hydrogel might be faster than biodegradation rate, as has been reported at our other previous paper (Konishi et al., 2003; Olson et al., 2003; Han et al., 2004; Chenite et al., 2000; Jeong et al., 1997). We expected that the chitosan hydrogel had great potential as a thermosensitive injectable delivery system for the local drug administration of cancer-killing agents without the severe systemic side effects including an immunotoxicity (Han et al., 2004).

To check the possibility of CH-DOX as a biocompatible and local cancer drug delivery system for future clinical chemoimmunotherapy, first of all, we evaluated the antitumor effects of CH-DOX itself by measuring tumor volumes after injection via various routes. Among them, intratumoral injection of CH-DOX exhibited the strongest antitumor effects (Fig. 1). The antitumor effects of the intratumoral injection of free DOX maintained for 7 days after injection. On the other hand, the effectiveness of the intratumoral injection of CH-DOX was sustained more than 9 days after injection, suggesting that the chitosan hydrogel system might prolong the exposure of tumor cells to DOX as compared with intratumoral administration of free DOX. To understand a precise action mechanism of CH-DOX after intratumoral injection a pharmacokinetic study is needed.

We tried to further improve the therapeutic efficacy by combining CH-DOX with Vac-Sig/E7/LAMP-1. Although the combination of chemotherapy with immunotherapy offers a promising method for cancer treatment, it is necessary to determine the best synergistic effects of this systemic approach while avoiding the undesirable immunotoxicity that might be caused by the chemical agents. The rationale for the use of the hydrogel system for chemoimmunotherapy is that intratumoral delivery of anti-cancer drugs for local treatment may be more effective in treating cancer cells by increasing the local toxic effects with a minimal damage to the host immune systems. Therefore, the hydrogel system could reduce the systemic immunotoxicity of anti-cancer drugs, and could represent one of the best delivery systems of chemotherapeutic agents for chemoimmunotherapy to treat tumors (Olson et al., 2003). In addition to the injection route of the chemoimmunotherapeutic agent, the injection timing between vaccination and CH-DOX

administration is also crucial for determining the antitumor effects of chemoimmunotherapy. It is noteworthy that anti-cancer drugs can generate harmful effects on the viability of immune cells as well as a live or attenuated virus. The viability of live or attenuated virus such as Vac-Sig/E7/LAMP-1 could be affected by genotoxic cancer drugs. DOX is also a genotoxic drug, which can act as a potential anti-viral agent for several viruses (Sekizawa et al., 2003). As shown in Fig. 2a, vaccination of TC-1 bearing mice with Vac-Sig/E7/LAMP-1 at 0 or 3 days after treatment with CH-DOX failed to further reduce the tumor growth by vaccination. Although the systemic toxicity of anti-cancer drugs can be significantly reduced by using the hydrogel system for local delivery of cancer drugs, a vaccinia virus-based vaccine system seems to be highly sensitive to cancer drugs. DOX released from tumor might decrease the number of live Vac-Sig/E7/LAMP-1 locally or, possibly, systemically. In our unpublished data, we have found that less then 1×10^6 PFU of Vac-Sig/E7/LAMP-1/mouse, which was one tenth of the titer used in this study, was not sufficient to induce significant E7-specific IFN-γ-secreting $CD8^+$ T cell immunity. However, pre-vaccination with Vac-Sig/E7/LAMP-1 at 3 days before CH-DOX treatment avoided these anti-viral effects of DOX and, in turn, generated the strongest antitumor effects as shown in Fig. 3.

More importantly, the optimized combinational protocol of pre-vaccination and CH-DOX treatment elicited a greater number of E7-specific IFN-γ-secreting $CD8^+$ T cells as compared to pre-vaccination with CH as shown in Fig. 4. As noted in the literature, many of chemotherapeutic agents such as DOX, cyclophosphamide, gemcitabin, adriamycin and 5-fluorouracil slightly increased the antitumor immune response via cross-presentation of an apoptotic tumor body mediated by caspase activation (Lake and Van der Most, 2006; Nowak et al., 2006; Casares et al., 2005). However, we failed to observe a significant induction of antitumor immune response after the combination of CH-DOX and Vac-W.T. treatment in our experimental settings (Fig. 4a). Thus, the enhanced antitumor immunity of the combined therapy does not seem to be caused by a direct adjuvant effect of CH-DOX. On the basis of this observation, we propose that CH-DOX treatment may augment the antitumor immunity induced by the vaccinia viral vaccine through enhanced tumor cell death, resulting in the uptake of tumor antigen by antigen presenting cells (APCs) through an exogenous cross-presentation pathway, a process involving the presentation of an exogenous antigen through the MHC class I pathway to $CD8^+$ T cells (Kang et al., 2007). In our previous report, we have observed an increase in the number of tumor antigen presenting $CD11c^+$ DCs in draining lymph nodes of tumor-bearing mice and, accordingly, an increase of the tumor-specific $CD8^+$ T cell immunity in tumor-bearing mice treated with epigallocatechin gallate (EGCG), a major component of polyphenols known as cathechins that have antitumor activity, combined with a Sig/E7/LAMP-1 DNA vaccine in a dose-dependent manner (Kang et al., 2007). Taken together, our findings provide evidence that chemotherapy may aid in boosting antigen-specific tumor immunity via cross-presentation of dead tumor cells (Lake and Van der Most, 2006; Nowak et al., 2006). However, we did not rule out the possible immunological events other than CD8+ T cell immunity in developing the anti-cancer effect by CH-DOX. Some evidence regarding the immunomodulating effects by DOX, has been indicated that DOX induced the activation/differentiation of cells of the monocytic-macrophage type and stimulation of T cell responses (Ehrke et al., 1996), increased production of interleukin-1, interleukin-2, and TNF-*a*, and modulation of natural killer (NK) cell function (Ehrke et al., 1996). Further studies on the antitumor effects of the combined therapy are needed to confirm.

This strong antitumor effect continued, and survived all of the mice treated by the combined therapy up to 70 days after tumor challenge (Fig. 5). This long-term protection of chemoimmunotherapy seems to be highly associated with induction of tumor antigen-specific antitumor immunity by the Vac-Sig/E7/LAMP-1 vaccine, especially E7-specific $CD8^+$ T cell immunity. More than 50 of E7-specific $CD8^+$ T cell among 1×10^5 spleenocytes were existed in each of the survived mice at 70 days after tumor challenge (data not shown).

In this study, a thermosensitive chitosan hydrogel containing doxorubicin (CH-DOX) was used as a local delivery system of a chemotherapeutic agent for chemoimmunotherpay. The system exhibited a synergistic antitumor effect after combination with a vaccinia virus-based vaccine. This combination did not decrease but rather increased the tumor-specific $CD8^+$ T cell immunity primed by vaccinia virus-mediated vaccination. We believe that, this study is the first report demonstrating the use of a biodegradable hydrogel system as a cancer drug delivery system for the application of successful chemoimmunotherapy. It is hoped that, this study can be a foundation for a rational approach to improve the antitumor efficacy of chemoimmunotherapy using hydrogel-based cancer drug delivery systems.

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