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Morphine Tolerance Suppresses Anti-tumor Effect of a DNA Vaccine Encoding Calreticulin Linked to a Tumor Antigen

ABSTRACT

Although morphine has been widely used in cancer patient with pain, the question whether morphine tolerance could simultaneously promote tumor growth through an antigen-specific immunosuppressive effect remains to be investigated. We have shown that calreticulin linked with HPV type 16 E7 antigen (CRT/E7) enhances the antigen specific $CD8^+$ T cell immune responses and the *in vivo* anti-tumor effects on the E7-expressing tumor cell line (TC-1). Herein, we report that morphine-tolerant mice exhibited higher tumor growth rates and lower percentages of $CD4^+$ and $CD8^+$ T lymphocytes than morphine naïve mice. In the CRT/E7 DNA vaccinated mice, morphine tolerance decreased the numbers of E7-specific $CD8^+$ T lymphocytes and suppressed the associated anti-tumor effect more rapidly than saline (Can you put in the quantitative data here?). The anti-tumor effect of the CRT/E7 DNA vaccine was reduced only in mice receiving daily injection of 20 mg/kg/day morphine for 10 days (60% of tumor-free mice) but not in 10 mg or 5 mg/kg/day groups (100% of tumor-free mice). Both *in vivo* and *in vitro* results clearly demonstrate that morphine dose-dependently promotes tumor growth and reduces the anti-tumor effect of cancer vaccine by inducing the apoptosis of antigen-specific $CD8^+$ T lymphocytes. The immunosupression on cytotoxic T cell and antigen-specific DNA vaccine may warrant the potential risk of tumorigenesis among those who receive longterm treatment with high dose morphine.

Keywords: morphine, DNA vaccine, immunosuppression, T cell apoptosis

INTRODUCTION

Although morphine has been accepted as a safe medication with great analgesic efficacy, numerous studies reported that morphine is highly associated with the risk of immunomodulatory and immunosuppressive effects both *in vitro* and *in vivo* (1, 2). The mechanisms underlying morphine-induced immunosuppression, although not well delineated, may include direct actions on the cellular immunity system and indirect actions mediated via the central nervous system (3, 4). Animals receiving morphine on a chronic basis showed marked atrophy of the thymus and spleen, and the microscopic examination of these organs revealed a markedly decreased lymphocyte count (5). Morphine altered mitogen-stimulated T and B cell responses (6, 7), attenuated antigen-specific antibody production (8), reduced NK cell activity (9), and suppressed concanavalin A -stimulated Ca^{2+} influx in both T and B lymphocytes (10). Polymorphonuclear cells and monocytes, when treated with morphine also showed decreased phagocytic and bacterial killing properties (11). Most of the previously observed immunosuppressive effects were induced by acute administration of the morphine and focused primarily on the innate immunity. However, the impact of long-term morphine treatment or morphine tolerance on the cellular immunity, especially on the antigen-specific $CD8^+$ lymphocytes, remains to be elucidated.

As immunity is closely associated with tumor growth and progression, antigen-specific cancer immunotherapy recently reveals an attractive approach for cancer treatment. It represents the ideal cancer treatment in being able to eradicate systemic tumors at multiple sites in the body with discrimination between neoplastic and non-neoplastic cells (12, 13). We have demonstrated that the anti-tumor effects of cancer vaccine, such as CRT/E7 DNA vaccine, can be produced through enhancing specific $CD8^+$ T lymphocyte immunity in mice (14). In this study, we address whether morphine-tolerant mice exhibit reduced anti-tumor effects of CRT/E7 DNA vaccine through selective immunosuppression of antigen-specific $CD8^+$ T lymphocytes. We found that the speed of tumor growth was higher in morphine-tolerant mice than morphine-naïve mice while the percentages of $CD4^+$ and $CD8^+$ T lymphocytes also decreased in morphine-tolerant mice. Long-term morphine injection of CRT/E7-DNA vaccinated mice resulted in decreased numbers $CD8^+$ cytotoxic T lymphocytes. As a result, the suppression of anti-tumor effects of DNA vaccinated in morphine-tolerant mice is dose-dependently related to morphine. Furthermore, we also discovered the mechanisms of morphine to reduce the anti-tumor effects of CRT/E7-DNA vaccine in vaccinated mice might be through the suppression of antigen-specific $CD8^+$ T lymphocytes proliferation and the promotion of apoptosis of these cells. Our results indicate that long-term use of morphine dose-dependently suppresses the function of specific $CD8^+$ T lymphocyte, promotes *in vivo* tumor growth, and leads

to the reduction of the anti-tumor effect of antigen-specific DNA vaccine.**MATERIAL AND METHODS**

Plasmid DNA Constructs and Preparation

The generation of pcDNA3-CRT/E7 has been described previously and kindly provided by Dr. TC. Wu (Johns Hopkins Medical Institutes) (14). Plasmid constructs were confirmed by DNA sequencing.

Cell Line

The production and maintenance of TC-1 cells has been described previously (15). HPV-16 E6, E7 and *ras* oncogene were used to transform primary lung epithelial cells of C57BL/6 mice. This tumorigenic cell line was named TC-1. TC-1 cells were grown in RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, 50 units/ml penicillin/ streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 2mM nonessential amino acids and 0.4 mg/ml G418 at 37^{o}C with 5% CO_2. On the day of tumor challenge, tumor cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS) and finally resuspended in 1X HBSS to the designated concentration for injection.

DNA Vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination were performed using a helium-driven gene gun (BioRad Laboratories Inc., Hercules, California, USA) according to a protocol described previously (16). CRT/E7 DNA-coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (BioRad Laboratories Inc.) with a discharge pressure of 400 psi.

Morphine injection

The mice would receive PBS or morphine sulfate (concentration: 1 mg/ml) with intraperitoneal injection (volume according to mice weight, 10,5, or 2.5 mg/kg) twice daily for 10consecutive days ((17-19)). The assessment of morphine tolerance and dependence were evaluated as mentioned later.

Assessment of morphine tolerance and dependence

The development of tolerance to antinociceptive effect of morphine were assessed on days 1, 3 and 10 after morphine injection by the tail-flick test (20). Briefly, each mouse was first tested twice before drug administration and the reaction times were averaged to obtain a baseline. The intensity of the heat stimulus was adjusted so that the mouse will flick its tail after 2-4 seconds. A minimum of three trials was recorded for each mouse 45-60 min after

morphine injection. Development of morphine dependence was assessed from naloxone-precipitated withdrawal jumps. Immediately after the tail-flick test on day 10, the mice were injected with naloxone (2 mg/kg) intraperitoneally and was individually placed in a Plexiglass box (45 x 30 x 30 cm). The number of withdrawal jumps was recorded over a 20-min period.

Mice

6- to 8-week-old female C57BL/6J mice were purchased and kept in the animal facility of the school of Medicine, National Taiwan University. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

***In Vivo* Tumor Growth Experiments**

For evaluating the *in vivo* tumor growing kinetics in mice with or without morphine treatment, C57BL/6 mice (five per group) either received PBS or morphine sulfate (10, 5 or 2.5 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before tumor injection. TC-1 was prepared and injected into the right leg of the mice subcutaneously with $5x10^4$/mouse. Mice were monitored for evidence of tumor growth by palpation and measured the tumor diameter twice a week until they were sacrificed at day 60.

Cell surface marker staining and flow cytometric analysis

Splenocytes removed from naïve or CRT/E7 vaccinated groups of PBS or morphine-treated mice for different time intervals were immediately treated with cell surface marker staining using a previously described protocol (14). Cells will be then washed once in FACScan buffer and stained with PE-conjugated monoclonal rat anti-mouse CD4, CD8 or NK1.1 Ab and FITC-conjugated monoclonal rat anti-mouse CD3 Ab (PharMingen). The population of lymphocytes (CD3 (+)), helper T lymphocytes (CD4(+)CD3(+)), cytotoxic T lymphocytes (CD8(+)CD3(+)), NK cells (NK1.1(+)CD3(-)) from different groups of mice were analyzed by flow cytometry.

Intracellular Cytokine Staining and Flow Cytometry Analysis

Mice were immunized with 2 μg of the CRT/E7 DNA vaccines and received a booster with the same regimen 1 week later. Splenocytes were harvested 1, 2, 3, 4, or 5 weeks after the last vaccination, respectively. The mice received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before DNA immunization until the days before being sacrificed. Before intracellular cytokine staining, $3.5x10^5$ pooled splenocytes from each vaccinated group were incubated for 16 hours with either 1 μg/ml of E7 peptide (aa 49-57) containing an MHC class I epitope (21)

for detecting E7-specific $CD8^+$ T cell precursors. Cell surface marker staining of CD8 and intracellular cytokine staining for IFN-γ, as well as FACScan analysis, were performed using conditions described previously (22).

Enzyme-linked Immunoabsorbent Assay (ELISA) for Anti-E7 Antibody

For the detection of HPV16 E7-specific antibodies in the sera, a direct ELISA was used as described previously (23). Mice were immunized with 2 μg of the CRT/E7 DNA vaccines and received a booster with the same regimen 1 week later. Sera were prepared from mice on day 14, 21, 28, 35, or 42 days after immunization. The mice received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before DNA immunization until the days before being sacrificed. Briefly, a 96-microwell plate was coated with 100 μl of bacteria-derived HPV-16 E7 proteins (0.5 μg/ml) and incubated at 4°C overnight. The wells were then blocked with phosphate-buffered saline (PBS) containing 20% fetal bovine serum. Sera were prepared from mice on day 21 post-immunization, serially diluted in PBS, added to the ELISA wells, and incubated at 37°C for 2 hr. After washing with PBS containing 0.05% Tween 20, the plate was incubated with a 1:2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for 1 hr. The plate was washed, developed with 1-Step Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1 *M* H_2SO4. The ELISA plate was read with a standard ELISA reader at 450 nm.

***In Vivo* Tumor Protection Experiments**

For the tumor protection experiment, C57BL/6 mice (five per group) either received no vaccination or were immunized with 2 μg/mouse of CRT/E7 DNA with a gene gun. One week later, mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were subcutaneously challenged with $5xl0^4$ TC-1 cells/mouse in the right leg. The mice received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before DNA immunization until the days before being sacrificed. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60.

Statistical analysis

All data are expressed as mean ± SEM. Comparisons between experimental groups were performed with analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software (SPSS 9.0, SPSS Inc, Chicago, IL). Differences were considered significant if the probability value at difference was less

than 0.05.

RESULTS

Morphine promotes the tumorigenesis in immunocompetent naïve mice

We first determined if morphine influenced the rate of tumor growth. C57BL/6 mice either received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily beginning 10 days before tumor injection. TC-1 was prepared and injected into the right leg of the mice subcutaneously with $5x10^4$/mouse. As shown in **Figure 1**, the mean tumor sizes of morphine-treated mice were larger than those of PBS-treated mice beginning 11 days after tumor challenge ($p<0.01$)). Our results suggest that morphine increases the rate of *in vivo* tumor growth in naïve immunocompetent mice.

Morphine gradually decreases the percentages of $CD4^+$ and $CD8^+$ T lymphocytes in naïve mice

We then asked whether morphine would influence numbers of T lymphocytes. The mice first either received PBS or morphine sulfate as described earlier. Splenocytes removed from groups of PBS or morphine-treated mice for different time intervals (7, 14, 21, 28 or 35 days later) were immediately stained with PE-conjugated monoclonal rat anti-mouse CD4 or CD8 Ab and FITC-conjugated monoclonal rat anti-mouse CD3 Ab and analyzed by flow cytometry. The representative figures of FACScan analysis of the percentages of the $CD4^+$ T lymphocytes are shown in **Figure 2A**. The percentages of $CD4^+$ T lymphocytes in morphine-treated mice significantly less than those in PBS-treated groups from 7 days to 35 days after morphine injection (17.9±1.1 vs. 20.5±0.7, D7; 14.6±0.9 vs. 21.8±1.2, D14; 15.0±0.8 vs. 21.5±0.8, D21; 14.2±0.8 vs. 18.3±0.9, D28; 10.6±0.6 vs. 14.4±0.9, D35; $p<0.01$, AVONA) **(Figure 2B)**. The percentage of $CD8^+$ T lymphocytes in the morphine-treat group is similar to that of PBS-treat group after 7 days of morphine injection. However, the percentages of $CD8^+$ T lymphocytes decreased significantly in the morphine-treated group as compared to those in the PBS-treated group **s**ince 14 days after morphine injection (11.0±0.9 vs. 11.5±0.9 D7; 9.2±1.0 vs. 12.7±0.9 D14; 8.9±0.6 vs. 10. 2±0.9, D21; 9.0±0.7 vs. 10.5±0.8; D28; 7.2±0.5 vs. 10.6±1.1, D35; $p<0.01$, ANOVA) **(Figure 2C)**. Our results suggest that morphine decrease the percentages of $CD4^+$ and $CD8^+$ T lymphocytes in the spleen.

Morphine influence the quantities of antigen-specific $CD8^+$ T lymphocytes

We further evaluated whether morphine also influences the quantities of antigen-specific $CD8^+$ T lymphocytes generated from antigen-specific DNA vaccine. The mice first received PBS or morphine sulfate as described earlier until the days (not clear here) before being sacrificed. Mice were then immunized with 2 μg of the CRT/E7 DNA vaccines and received a booster with the same regimen 1 week later. Splenocytes were harvested 1, 2,

3, 4, or 5 weeks after the last vaccination, respectively. Before intracellular cytokine staining, splenocytes from each vaccination group were incubated for 16 hours with either 1 μg/ml of E7 peptide (aa 49-57). Cell surface marker staining of CD8 and intracellular cytokine staining for IFN-γ, as well as FACScan analysis, were performed. Representative figures of FACScan analysis of number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in PBS or morphine-treated mice receiving CRT/E7 DNA vaccination at different intervals are shown in **Figure 3A.** The numbers of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in morphine-treat group are significantly less than those in PBS-treated group since 21 days after last immunization (230.0±13.0 vs. 257.0±12.0 for D7; 181.5±7.5 vs. 203.5±5.5 for D14; 89.5±7.5 vs. 192.0±7.0 for D21; 39.0±4.0 vs. 152.5±7.5 for D28; 23.0±7.0 vs. 76.5±6.5 for D35; $P<0.01$, AVONA) **(Figure 3B)**. When defined the number of E7-specific IFN-γ-secreting $CD8^+$ T lymphocytes on D0 as 100%, the percentages of E7-specific IFN-γ-secreting $CD8^+$ T lymphocytes at different intervals in the PBS and morphine-treated groups. The percentage of E7-specific IFN-γ-secreting $CD8^+$ T lymphocytes of the morphine-treated group declined to less than 50% after 21 days of DNA immunization **(Figure 3C)**. However, the percentage IFN-γ-secreting $CD8^+$ T lymphocytes of the PBS-treated group persisted longer and declined to less than 50% after 35 days of DNA immunization.

We further evaluated that if the morphine would influence the titer of anti-E7 antibody of the DNA CRT/E7 vaccinated mice. Mice were immunized with 2 μg of the CRT/E7 DNA vaccines and received a booster with the same regimen 1 week later. Sera were prepared from mice on day 14, 21, 28, 35, or 42 days after immunization. The mice received PBS or morphine sulfate as described earlier. For the detection of HPV16 E7-specific antibodies in the sera, a direct ELISA was used as described in **Materials and Methods**. The titers of E7-specific antibody did not show significant difference between the PBS and morphine-treated groups at different intervals **(Figure 3D)**.

Morphine suppresses the anti-tumor effect of antigen-specific CRT/E7 DNA vaccine

We then evaluated that if the morphine would decrease the anti-tumor effect of CRT/E7 DNA vaccine to the TC-1 tumor cells (HPV16 E7-expressing tumor cells). The mice first either received PBS or morphine sulfate as described earlier. Then the mice were immunized with 2 μg/mouse of CRT/E7 DNA with a gene gun and challenged with $5xl0^4$ TC-1 cells/mouse. 100% of mice receiving PBS with CRT/E7 DNA vaccine remained tumor-free 60 days after TC-1 challenge. However, morphine-treated mice with CRT/E7 DNA vaccine developed tumor since 28 days after TC-1 challenge and only 40 % of mice in this group remained tumor-free 60 days after TC-1 challenge **(Figure 4A)**.

We further tested the anti-tumor effects of mice injected with different dosages of morphine and immunized with CRT/E7 DNA vaccine. The mice first received different

dosages of morphine (2.5, 5 or 10 mg/kg) since 10 days before DNA immunization and then immunized with 2 μg/mouse of CRT/E7 DNA. One week after last DNA immunization, the mice were challenged with $5xl0^4$ TC-1 cells/mouse. As shown in **Figure 4B**, 100% of mice receiving 2.5 mg/kg or 5 mg/kg morphine and then immunized with CRT/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. However, only 40% of the mice receiving 10 mg/kg morphine injection and then CRT/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. Our results show that high-dose of morphine decrease the antigen-specific anti-tumor effect of naked DNA vaccine. And the loss of anti-tumor effect was observed in long-term morphine treatment.

DISCUSSION

We first observed that morphine-tolerant mice exhibited higher tumor growth rates and lower percentages of $CD4^+$ and $CD8^+$ T lymphocytes as compared with those in mice without morphine treatment. While HPV16 E7 antigen-specific DNA vaccine could prevent tumor growth in morphine-naïve mice, morphine was capable of influencing the kinetic changes of E7-specific $CD8^+$ cytotoxic T lymphocytes and reducing the anti-tumor effects of antigen-specific DNA vaccine in a dose-dependent manner. Our *in vitro* studies further demonstrated that morphine inhibited the proliferation and promoted the apoptosis of E7-specific T lymphocytes.

Antigen-specific cytotoxic $CD8^+$ T lymphocytes play an important role in the anti-tumor effect. Tumor-associated antigens are present in several malignancies and represent promising targets for immunotherapeutic approaches. Antigen-specific $CD8^+$ T-cells are an important component of immunosurveillance in malignant diseases as they recognize, target, and kill tumor cells (25-27). Calreticulin (CRT) has demonstrated the ability to enhance MHC class I presentation to link with HPV type 16 E7 antigen. We previously explored the linkage of CRT to a model tumor antigen, Human papilloma virus type 16 (HPV16) E7, for the development of a DNA vaccine. We found that mice vaccinated intradermally with CRT/E7 DNA exhibited a dramatic increase in E7-specific $CD8^+$ T cell precursors and an impressive anti-tumor effect against E7-expressing tumors compared with mice vaccinated with wild-type E7 DNA. Besides, we also proved that CRT/E7 generated a significant $CD8^+$ T cell (neither $CD4^+$ T cell nor NK cell) –dependent protective effect against subcutaneous TC-1 tumors (14). The CRT/E7 DNA vaccine, TC-1 tumor cell line and the murine tumor model provide a good system for evaluating the influence of long-term use of morphine on antigen-specific $CD8^+$ T lymphocytes and their *in vivo* anti-tumor effects.

Morphine suppresses the function and the anti-tumor effect of antigen-specific cytotoxic $CD8^+$ T lymphocytes. Morphine has been reported to modulate the function of monocytes through opiate receptors of lymphocyte (28-30), indirectly via opiate receptors in the central nervous system, or by activating the

hypothalamic-pituitary-adrenal axis to secret immunosuppressive glucocorticoids (31, 32) The numbers of E7-specific $CD8^+$ T lymphocytes in morphine-treated group decreased significantly after vaccination from D7 to D35 as compared with those in PBS-treated mice in this investigation **(Figure 3)**. Besides, morphine-treat CRT/E7 DNA vaccinated mice had lower rate of anti-tumor effect as compared to CR/E7 DNA vaccinated mice without morphine treatment **(Figure 4A)**. Our results show that morphine could directly decrease the number and suppress the anti-tumor effect of antigen-specific T lymphocytes. These observations supported the hypothesis that morphine can modulate immune function in mice with morphine addiction or long-term treating (33).

Morphine inhibits the proliferation of T lymphocytes and induces the apoptosis of T lymphocytes. Singhal et al. evaluated the effect of morphine on the apoptosis of Jurkat cells (T immortalized cell line) and freshly isolated human T lymphocytes (34). They observed that morphine promoted apoptosis of both the Jurkat cells and the freshly isolated T lymphocytes in a dose-dependent manner. Their work supports the hypothesis that morphine could directly compromise the function of T lymphocytes by enhancing apoptotic pathway. Our *in vitro* study also showed that long-term injecting morphine to mice could suppress the proliferation **(Figure 5B)** and increase the apoptosis of E7-specific $CD8^+$ T lymphocytes **(Figures 6B and 7).** Morphine might directly compromise immune function by enhancing the apoptosis of T lymphocytes and decrease their number.

The role of opioid receptor in the morphine-triggered lymphocyte apoptosis has been thoroughly discussed (34). Our data revealed that morphine could inhibit the proliferation of E7-specific $CD8^+$ T cells **(Figure 5A)** and naloxone could rescue the inhibitive effects of morphine on these T lymphocytes **(Figure 5B)**. Both lymphocytes and macrophages have been demonstrated to express opioid receptors (35, 36). The direct effect of morphine on the mononuclear phagocyte system and on the specific cellular immune system has been proved about morphine-induced immunomodulation (37, 38). Naloxone, an opiate receptor antagonist, has been proved to attenuate morphine-induced effects (39, 40) or reverse the toxic signs in morphine intoxication (41, 42). Nalaxone was demonstrated to attenuate morphine-induced T lymphocyte apoptosis (34). Our observation demonstrates that morphine might be through the opiate μ receptor to suppress the function of T lymphocyte and then to promote tumor growth and to influence the anti-tumor effects of antigen-specific DNA vaccine in our mice model.

The generation of antibodies would not be suppressed by morphine. Long-term use of morphine injection did not influence the antibody titers in mice vaccinated with CRT/E7 DNA vaccine in our survey **(Figure 3D).** Bhargava et al. indicated that B-Cell proliferation was suppressed in morphine-tolerant but not in morphine-abstinent mice (19). Thus it might imply that the influence of morphine on the

anti-tumor effects of antigen-specific DNA vaccine would not be related to B lymphocyte function and the production of antibody in our observation. However, if long-term use of morphine would influence the production of antibody from the B cells need to be surveyed in further study.

Bax and Bcl-2 molecules involve the apoptotic process of T lymphocytes. Apoptosis is mediated through at least three major pathways that are regulated by the death receptors, the mitochondria and the ER (endoplasmic reticulum). In most cells, these pathways are controlled by the Bcl-2 family of proteins that can be divided into anti-apoptotic and pro-apoptotic members (43). Bax and Bcl-2 are prototypical members of a large family of Bcl-2-like proteins that play a key role in regulating programmed cell death in many cell types (44).Bax and Bcl-2 have been demonstrated to play a role in the effector phase of cell death in lymphocyte (45). Singhal, et al. observed that morphine could enhanced expression of Bax and decreased expression of Bcl-2 in the Jurkat cells and morphine-induced T lymphocyte apoptosis might be mediated through an altered Bax/Bcl-2 ratio (34). Our survey also noted that enhanced Bax expression and decreased expression of Bcl-2 in the E7-specific $CD8^+$ T lymphocytes were correlated with the concentration of morphine (**Figure 7**). It implicated that morphine induces the apoptosis of T lymphocytes thorough the two Bcl-2 and Bax apoptosis-related molecules.

Opioid addiction is a very perplexed social and medical issue all over the world. Although physiological responses to morphine-tolerance and physical dependence have been thoroughly elucidated, their putative adverse effects on the tumor genesis have rarely been reported. In this study, the mice rendered tolerant and dependent state with different doses of morphine (2.5, 5, or 10 mg/kg) for more than 10 consecutive days is a reliable animal model for opioid addiction in the clinical settings. The CRT/E7 DNA immunized mice, only when injected with high dose of morphine (10 mg/kg), lost partial anti-tumor effect. In the human model, morphine tolerance and dependence frequently occur in the cancer patients with long-term morphine injection for cancer pain and in the opioids addicts. The immunosuppressive effects of morphine addiction on the tumorigenesis in human are much more complex than in animals. Many other confounding factors would affect the tumor growth in morphine tolerance or dependence patients, not only the immunosuppressive effects of morphine addiction. The immunosuppressive effects of long-term morphine treatment on the tumor progression always are the challenging issue to be elucidated. We definitely indicated that long-term high-dose morphine treatment does suppress cellular immune function and promote tumor growth through the interaction with apoptosis and proliferation of antigen-specific $CD8^+$ T lymphocytes in our mice tumor growth model. Recently there is a tendency to reduce the dose of morphine for cancer pain treatment by adding other drug (such as ketorolac) to morphine regimen (46, 47). Further study should be implemented to explore that if these

new regimens (such as combining morphine with the other medicines) for controlling cancer pain will reduce the immunosuppressive effects and then do less harmful effect on tumor progression or growth.

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FIGURE LEGENDS

Figure 1. *In vivo* measurement of tumor growth in mice with or without morphine injection. C57BL/6 mice (five per group) either received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before tumor injection. TC-1 was prepared and injected into the right leg of the mice subcutaneously with $5x10^4$/mouse. *Note:* The mean tumor sizes of mice received morphine injection were larger than those of mice received PBS injection.

Figure 2. Flow cytometry analysis of the kinetic changes of the percentages of $CD4^+$ and $CD8^+$ T lymphocytes mice either injected with PBS or morphine. The mice first either received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection twice daily. Splenocytes removed from groups of PBS or morphine-treated mice for different time intervals (7, 14, 21, 28 or 35 days later) were immediately treated with cell surface marker staining. Cells were then washed once in FACScan buffer and stained with PE-conjugated monoclonal rat anti-mouse CD4 or CD8 Ab and FITC-conjugated monoclonal rat anti-mouse CD3 Ab and analyzed by FACScan. **(A) The representative figures of FACScan analysis of the percentages of the $CD4^+$ T lymphocytes. (B) Percentages of $CD4^+$ T lymphocytes in groups of PBS and morphine-treated mice at different intervals.** *Note:* The percentages of $CD4^+$ T lymphocytes in morphine-treated mice significantly decreased than those in PBS-treated groups from 7 days to 35 days ($p<0.01$, AVONA). **(C) Percentages of $CD8^+$ T lymphocytes in groups of PBS and morphine-treated mice at different intervals.** *Note:* The percentages of $CD8^+$ T lymphocytes in morphine-treated mice significantly decreased than those in PBS-treated groups since 14 days after injection. ($p<0.01$, AVONA).

Figure 3. The changes of immunologic responses in mice treated with morphine and received CET/E7 DNA vaccination. The mice first received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before DNA immunization until the days before being sacrificed. Mice were then immunized with 2 μg of the CRT/E7 DNA vaccines and received a booster with the same regimen 1 week later. Splenocytes were harvested 1, 2, 3, 4, or 5 weeks after the last vaccination, respectively. Before intracellular cytokine staining, $3.5x10^5$ pooled splenocytes from each vaccination group were incubated for 16 hours with either 1 μg/ml of E7 peptide (aa 49-57) containing an MHC class I epitope (21) for detecting E7-specific $CD8^+$ T cell precursors. Cell surface marker staining of CD8 and intracellular cytokine staining for IFN-γ, as well as flow cytometry analysis, were performed using conditions described earlier. **(A) Representative figures of FACScan analysis. (B) Number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in PBS or morphine-treated mice receiving CRT/E7 DNA vaccination at different intervals.** Data are expressed as mean number of E7-specific IFN-γ-secreting $CD8^+$

T cell precursors per $3.5x10^5$ splenocytes ± SEM. ***Note:*** The numbers of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in morphine-treat group are significantly less than those in PBS-treated group since 21 days after last immunization.($P<0.01$, AVONA). **(C) The percentages of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in PBS or morphine-treated mice receiving CRT/E7 DNA vaccination at different intervals.** ***Note:*** The decreasing slope of the morphine-treat group declines quicklier than that of PBS-treated group ($p<0.01$, AVONA).**(D) E7-specific antibody responses in PBS or morphine-treated mice receiving CRT/E7 DNA vaccination at different intervals.** ***Note:*** The titers of E7-specific antibody did not show significant difference between the PBS and morphine-treated groups.

Figure 4. (A) ***In vivo*** **tumor protection experiments in mice vaccinated with CRT/E7 DNA vaccine with or without morphine injection.** To evaluate the influence of morphine on the antitumor effect of DNA vaccine, the mice first either received PBS or morphine sulfate (10 mg/kg) with intraperitoneal injection (total 200 μl) twice daily since 10 days before DNA immunization and until the days before being sacrificed. Then the mice were immunized with 2 μg/mouse of CRT/E7 DNA with a gene gun and challenged with $5xl0^4$ TC-1 cells/mouse. *Note:* 100% of mice receiving PBS only and CRT/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. However, morphine-injected mice receiving CRT/E7 DNA vaccination developed tumor since 28 days after TC-1 challenge and only 40 % of the in this group remained tumor-free 60 days after TC-1 challenge. **(B) The anti-tumor effects of mice injected with different dosages of morphine and immunized with CRT/E7 DNA vaccine.** The mice first either received different dosages of morphine (2.5, 5 or 10 mg/kg) since 10 days before DNA immunization and then immunized with 2 μg/mouse of CRT/E7 DNA. One week after last DNA immunization, the mice were challenged with $5xl0^4$ TC-1 cells/mouse. *Note:* 100% of mice receiving 2.5 mg/kg or 5 mg/kg morphine and then immunized with CRT/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. However, 60% of the mice receiving 10 mg/kg morphine injection and then CRT/E7 DNA vaccination developed tumor within 60 days after TC-1 challenge.