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Combined Treatment of an Intratumoral Injection of Dendritic Cells and Systemic Chemotherapy (Paclitaxel) for Murine Fibrosarcoma

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A novel combined treatment of conventional chemotherapy with an intratumoral injection of syngeneic dendritic cells (DCs) has emerged as a potent cancer treatment strategy. In this study, we evaluated the synergistic effect of an intraperitoneal (i.p.) injection of a chemotherapeutic drug, paclitaxel, and an intratumoral (i.t.) injection of syngeneic bone marrow-derived DCs for the treatment of pre-existing fibrosarcoma. Subcutaneous tumors were established using MCA102 fibrosarcoma cells in syngeneic C57BL/6 mice. The results demonstrated that the combined treatment of paclitaxel chemotherapy and the injection of DCs led to complete tumor regression, in contrast to only partial eradication of the tumors with chemotherapy or DCs alone. Furthermore, the tumor-free mice were able to resist a repeat challenge with the same type of tumor. These findings suggest that a combination therapy of systemic chemotherapy along with the intratumoral administration of DCs is a potent treatment strategy for fibrosarcoma.

Key Words: Dendritic cells, paclitaxel, immunotherapy

INTRODUCTION

Dendritic cells (DCs) are the most effective antigen-presenting cells recognized by T cells, and they have the potential to induce tumor-specific immune responses leading to tumor rejection. DCs capture tumor cells, then generate tumor-specific cytotoxic T lymphocytes (CTLs) from naive T cells to fight against tumor cells.[1-3] Although tumors have immunogenic epitopes on their surface that can be recognized by the host immune system, failure to produce an appropriate immune response to these antigens results in uncontrolled tumor growth in cancer patients. Immune tolerance to an established tumor is due to the low immunogenicity of the tumor and/or defects in the host immune system.[4-6]

Several published trials investigating the immune system's ability to overcome tumor tolerance involve the development of a tumor vaccination that injects DCs, generated and loaded with tumor antigens *ex vivo* into cancer patients.[7-11] Using these approaches, *ex vivo* manipulation of the DCs is required for the cells to acquire and present tumor-specific antigens. To avoid the requirement of an *ex vivo* antigen loading process, some researchers have suggested the administration of a DC injection after systemic chemotherapy; this strategy is based on the ability of DCs to ingest apoptotic tumor cells and acquire tumor-associated antigens, which induces class I-restricted CTLs *in vitro*.[12-15] These trials showed that the antitumor effect was significantly more effective when both chemotherapy and an injection of DCs were administered.

Paclitaxel is an anticancer drug that is effective

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against a variety of cancers. Paclitaxel binds to tubulin, retards microtubule depolymerization, impairs mitosis, blocks cell cycle progression, and facilitates apoptosis.[16] Various chemotherapeutic drugs are currently being used for combination therapy studies of DCs and chemotherapy. Paclitaxel was tested for use as a treatment for advanced stage breast cancer in an animal model.[15] Recently, it was reported that the cytotoxic effect of paclitaxel on DCs was minimal.[17] In our study, we observed an antitumor effect associated with a DC therapy after low-dose paclitaxel administration for the treatment of murine fibrosarcoma. The results indicate that the intratumoral (i.t.) administration of DCs after low-dose chemotherapy via an intraperitoneal (i.p.) injection results in potent and specific antitumor immunity, without an *ex vivo* antigen loading process, for the treatment of fibrosarcoma.

MATERIALS AND METHODS

Tumor cell culture

MCA102 is a murine fibrosarcoma cell line of C57BL/6 mouse origin. This cell line was maintained in a complete medium that contained RPMI 1640 medium (Gibco-BRL, Rockville, MD, USA) plus 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL) and 100 U/mL penicillin (GIBCO-BRL) in a 5% CO_2 incubator at 37℃ to a subconfluent state in 75 cm^2 plates. After reaching subconfluency, cells were rinsed twice with phosphate-buffered saline (PBS) or RPMI 1640. For detachment, cells grown to confluency were treated with 0.05% trypsin (Gibco-BRL) and 0.53 mM EDTA. For the *in vivo* inoculation, tumor cells were washed three times and resuspended in PBS.

Murine DC culture

Primary DCs were obtained from mouse bone marrow precursors. Murine bone marrow cells were harvested from femurs and tibias and then plated in complete RPMI1640 containing recombinant murine GM-CSF (10 ng/mL) and recombinant murine IL-4 (10 ng/mL). On day two, non-adherent granulocytes were gently removed, and fresh medium with GM-CSF and IL-4 was added. On day four, loosely adherent cells were dislodged and replated. On day six of the culture, immature DCs and non-adherent proliferating aggregates were collected and the maturation status and percentage of DCs were verified by flow cytometry with five surface markers (CD11c, CD44, CD40, CD80, and MHC class II antigen I-Ad), showing the purity of DCs to be >65%.

MTT assay

The number of viable cells at the end of the culture was quantified by an MTT assay. The tumor cells and DCs in their own complete media were incubated for 48 hr in 24-well plates containing serial dilutions of the chemotherapeutic drugs in a total volume of 1 mL/well. The concentrations of the dilutions of etoposide, doxorubicin and ifosfamide used were: 0.005, 0.01, 0.05, 0.1, 0.5 and 1 ug/mL. The concentrations of the dilutions of paclitaxel used: were 0.005, 0.01, 0.05 0.1, 1, 5, 10 and 50 ug/mL. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma), was dissolved in PBS at 5 mg/mL. Following exposure to the chemotherapeutic drugs, a 1 : 10 diluted MTT solution (0.5 mg/mL) was added to the assay wells. The plates were incubated at 37℃ for 24 hr. Viable cells generated insoluble crystals, but DCs were floating and loosely attached on the surface of the culture plates. Therefore, 200 uL/well 10% SDS solution containing 0.01 N HCl was directly added into the wells to avoid the potential loss of cells and to dissolve the insoluble crystal generated by the cells. After 24 hr, the amount of converted MTT was quantified in an ELISA reader using a 570 nm test and 650 nm reference wavelengths. The results are expressed as the mean ± SD of absorbance.

Animals

Female C57BL/6 mice were purchased from Dae Han Bio Link (Korea). The mice were allowed to adjust to their environment for one week, and DC isolation was performed at six to eight weeks of age, and tumor inoculation was performed at eight to ten weeks of age.

Combined treatment of intratumoral DC injection and chemotherapy

For the fibrosarcoma MCA102 tumor model, naive C57BL/6 mice were inoculated s.c with 1.5 $\times 10^5$ MCA102 cells in the upper right flank. On day nine, when the tumor size reached 3-5 mm in diameter, paclitaxel (5 mg/kg) was administered i.p. to the mice and then the same treatment was given on the 11th and 13th days. DCs were injected i.t. once daily four times, on days 10, 12, 14 and 18 (2×10^6 cells/mouse for each injection in 50 ul of PBS). Mice were divided into four groups (four mice per group): untreated control mice, mice treated with DCs alone, mice treated with paclitaxel alone, and mice treated with paclitaxel plus DCs. For all animal experiments, the tumor size was measured biweekly using a caliper and expressed as the product of the maximal perpendicular diameter and height (mm^3).

In vivo analysis of cell death by TUNEL stain

The free 3-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. After a wash with PBS, tissue slides were fixed in 10% paraformaldehyde. The TUNEL assay was conducted according to the manufacturer's instructions (TACS In Situ Apoptosis Detection kit, Trevision, USA).

Secondary tumor challenge in tumor-free mice

The persistence of tumor-specific immunity in the mice treated by combination therapy was determined at day 74, 146 days after first tumor inoculation. Two mice showing a complete regression of MCA102 fibrosarcoma tumors were given a second s.c. tumor challenge. MCA102 fibrosarcoma tumor cells (2×10^5 cell each in 50 uL PBS) were implanted into the left upper flank of each mouse, opposite to the first injection site. The same number of tumor cells was also injected into age-matched control mice. These mice were followed for survival and measurement of tumor size.

RESULTS

Isolation of bone marrow-derived dendritic cells and phenotyping

Primary DCs were obtained from syngeneic mouse bone marrow precursors from femurs and tibias. On day six of the culture, in the presence of GM-CSF and IL-4, immature DCs were collected and their phenotypes were verified by flow cytometry (Fig. 1). As shown in Fig. 1, almost no expression of maturity surface markers CD40 and CD80 was detected, indicating that the DCs were in an immature state. Flow cytometry data with three other surface markers, I-Ad, CD11c, and CD44, showed the purity of the DCs to be >65%.

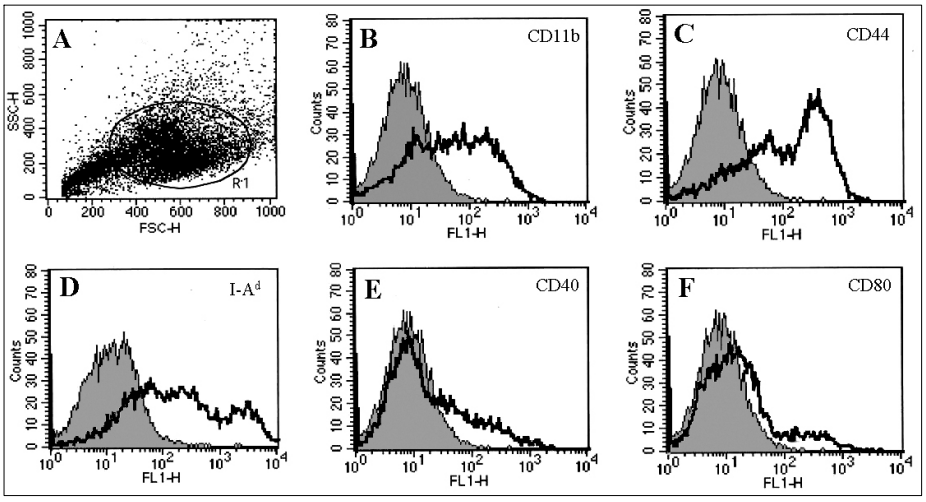
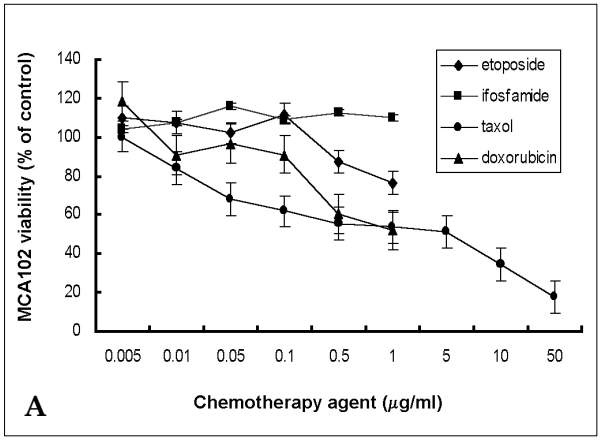


Fig. 1. Phenotyping of cultured dendritic cells. Primary DCs were obtained from syngeneic mouse bone marrow precursors by culturing in the presence of GM-CSF and IL-4; their phenotypes were verified by flow cytometry. The results showed that the DCs isolated were CD11c (B), CD44 (C), and I-Ad (D) positive. Both CD40 (E) and CD80 (F) expression was very low.

In vitro cytotoxic effect of paclitaxel on murine fibrosarcoma cells and DCs

In order to find an effective chemotherapeutic agent for MCA102 tumor cells, the cytotoxicity of etoposide, ifosfamide, doxorubicine, and paclitaxel were tested by the MTT assay *in vitro*. Among these, paclitaxel had the most significant cytotoxic effect on MCA102 tumor cells (Fig. 2A). In addition, the cytotoxic effect of paclitaxel on DCs was also tested and shown to have a lower cytotoxic effect than that on MCA102 (Fig. 2B). These results suggested that paclitaxel would be an effective chemotherapeutic agent for this study.



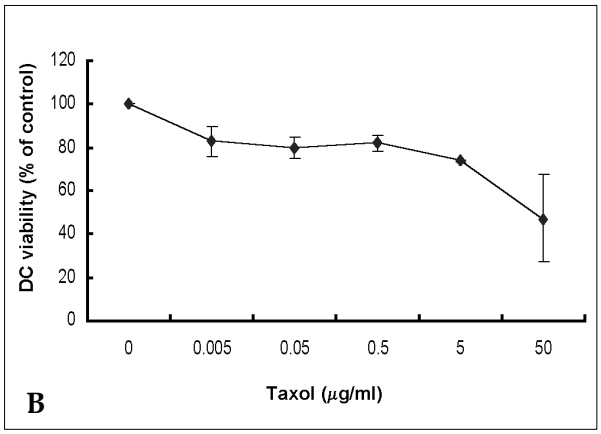


Fig. 2. *In vitro* cytotoxic effect of paclitaxel on MCA102 murine fibrosarcoma cells and DCs. The cytotoxicity effects of etoposide, ifosfamide, doxorubicine, and paclitaxel on MCA102 tumor cells (A) and the cytotoxicity effects of paclitaxel on DCs (B) were measured by an MTT assay *in vitro*. Paclitaxel was found to have the most profound cytotoxic effect on MCA102 tumor cells.

Significant regression of tumor growth in the combined treatment group in contrast to control single treatment groups

To determine whether systemic paclitaxel administration by i.p. together with an i.t. injection of DCs has a synergistic effect compared to chemotherapy or a DC treatment alone, a well-characterized tumor model (MCA102 sarcoma in C57BL/6 mouse) was evaluated. Paclitaxel chemotherapy was initiated i.p. when the tumor size was approximately 4-5 mm^2, and i.t. administration of DCs was initiated one day after the first dose of chemotherapy. A detailed injection schedule is described in the Materials and Methods section. As shown in Fig. 3, the tumors grew rapidly in the untreated control mice. The mice treated with paclitaxel alone and DCs alone showed a modest suppression of tumor growth. In contrast, the mice treated with combined systemic paclitaxel administration plus an i.t. injection of DCs showed a significant suppression of tumor growth. These results strongly suggest that combined systemic paclitaxel chemotherapy plus an i.t. injection of DCs has greater suppressive activity on MCA102 sarcoma growth than either paclitaxel or DC treatment alone *in vivo*. The representative images of the actual tumor sizes in each group on day 15

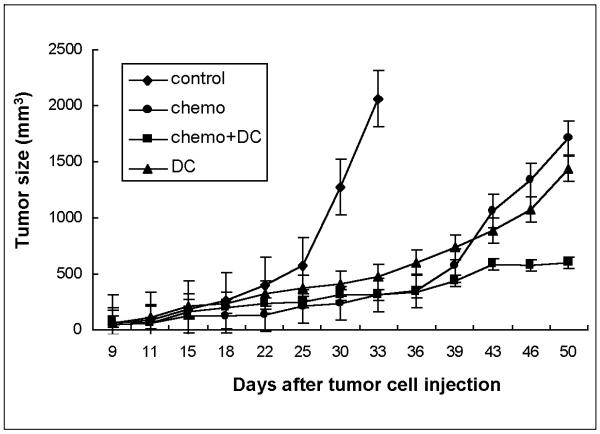


Fig. 3. Significant regression of tumor growth in the combined treatment group in contrast to the control groups. Using a well-characterized tumor model (MCA102 sarcoma in C57BL/6 mouse), a synergistic effect of a systemic paclitaxel administration by i.p. together with an i.t. injection of DCs was evaluated in comparison to chemotherapy or DC treatment alone. The data represent the mean ± SD of three independent experiments.

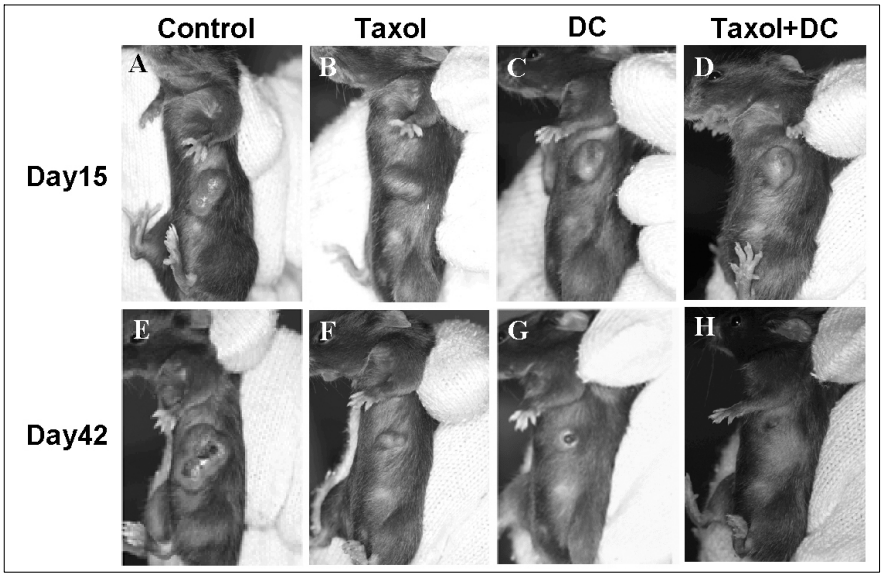


Fig. 4. Representative photographs of animals showing the tumor masses 15 days and 42 days after the tumor cell injection. The mouse treated with the combined therapy showed the most significant suppression of tumor growth. (A, B, C, and D) mice treated with medium, paclitaxel, DC, or paclitaxel plus DC, respectively, on day 15. (E, F, G, and H) mice treated with medium, paclitaxel, DC, or paclitaxel plus DC, respectively, on day 42.

and day 42 post-tumor cell injection are shown in Fig. 4.

Apoptosis of MCA102 tumor cells in tumor tissue after the combined treatment *in vivo*

To evaluate the apoptosis of MCA102 tumor cells in tumor tissue after each treatment, the TUNEL assay was performed with paraformaldehyde-fixed tissue specimens. Apoptotic cells stained brown were easily observed in the tumor tissues treated with the combination of paclitaxel and DCs or paclitaxel alone but not as readily observed in the tissues treated with DCs alone (Fig. 5). These results indicate that an i.p. injection of paclitaxel caused apoptosis of MCA102 tumor cells *in vivo*.

Immunohistochemical staining of tumor tissue after the combined treatment

In order to observe the migration of immune cells to the tumor mass *in vivo*, an immunohistochemical stain was performed using anti-CD11c. When the border areas between the tumor tissue and normal tissue were examined, more immune cells, such as macrophages and neutrophils, were detected in frozen sections from the combined treatment group, as compared to the control group to which only cell culture medium was injected (Fig. 6).

Persistent antitumor memory after the combined treatment

To further evaluate the memory of antitumor immunity *in vivo* after the combined treatment of paclitaxel plus DCs, a second challenge of the same MCA102 tumor cells was delivered to the mice, in which the tumor had completely regressed. As shown in Fig. 7, in three experimental mice, a very small tumor appeared around day nine after the injection, but disappeared quickly. In contrast, as observed in the previous control experiments, tumors formed in the control mice after a second challenge. These results suggested that the tumor-free mice were able to resist a repeat challenge with the same type of tumor.

DISCUSSION

The present study demonstrates that a combination therapy of conventional paclitaxel chemotherapy and an i.t. injection of DCs is effective against fibrosarcoma in a C57BL/6 mouse model of flank fibrosarcoma that was syngeneic to the animal. The data showed that the combined treatment resulted in the complete suppression of tumor growth, whereas the single treatments of chemotherapy or DC injection alone generated only a partial regression of the tumor. In addition, the combined therapy produced tumor-specific

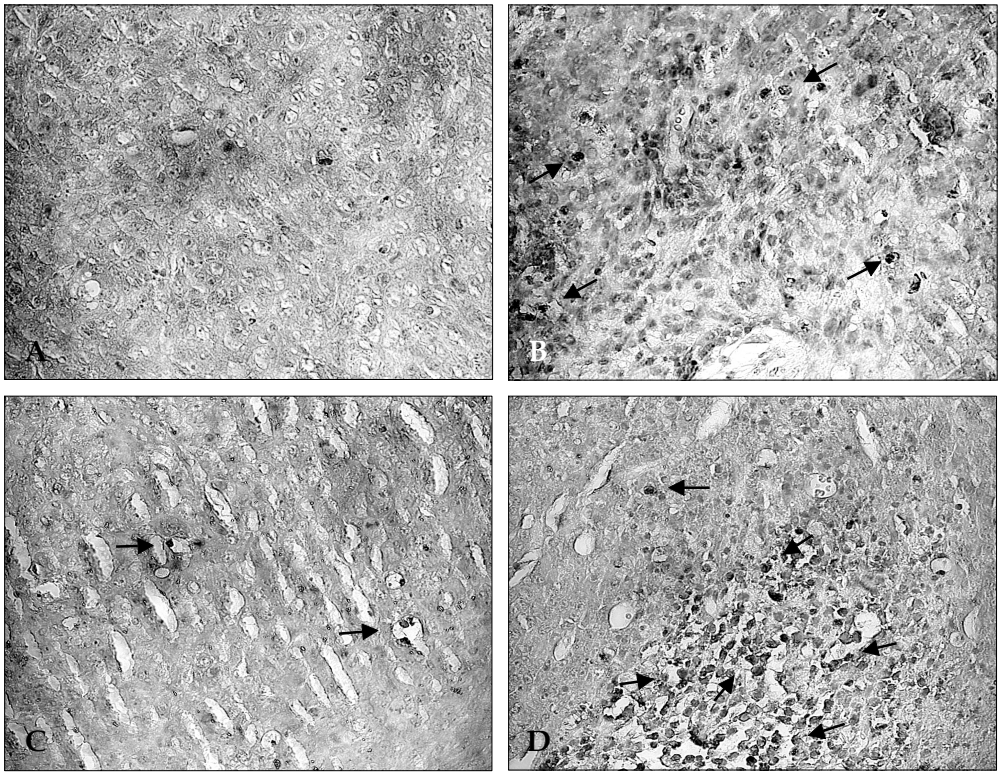


Fig. 5. Determination of apoptosis of MCA102 tumor cells in the tumor tissues by the TUNEL assay. Apoptotic cells stained brown were easily observed in the tumor tissues treated with the combined treatment (D) or paclitaxel treatment alone (B) but not in tissues treated with DC treatment alone (C) or medium injection (A). Arrows indicate the apoptotic cells.

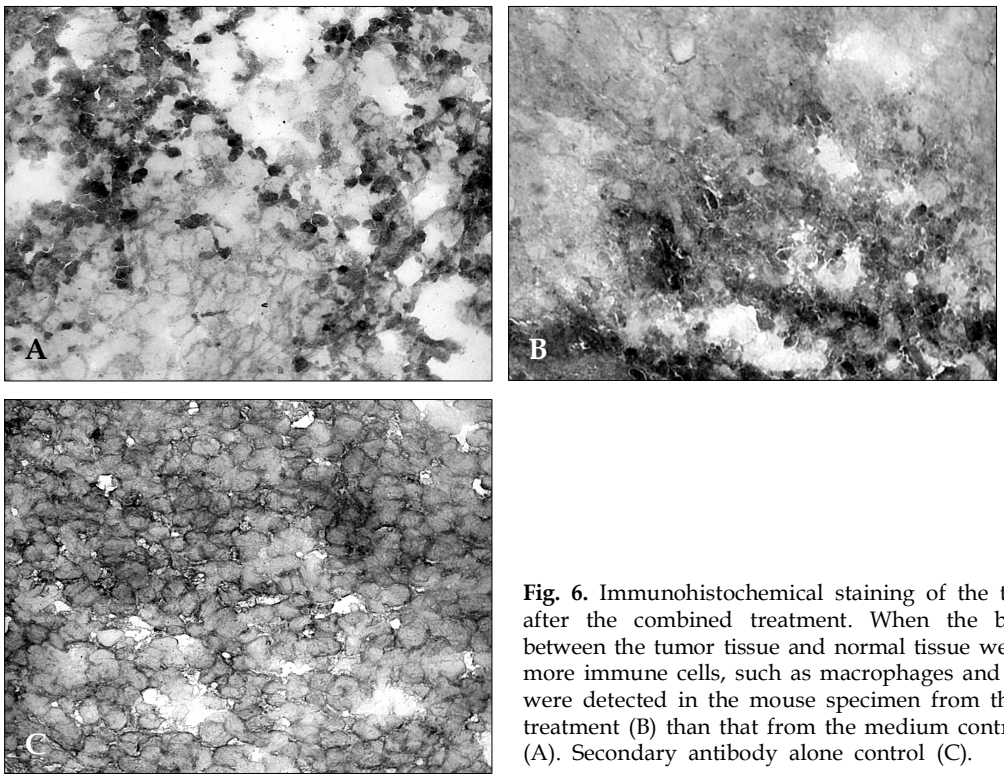


Fig. 6. Immunohistochemical staining of the tumor tissue after the combined treatment. When the border areas between the tumor tissue and normal tissue were observed, more immune cells, such as macrophages and neutrophils, were detected in the mouse specimen from the combined treatment (B) than that from the medium control specimen (A). Secondary antibody alone control (C).

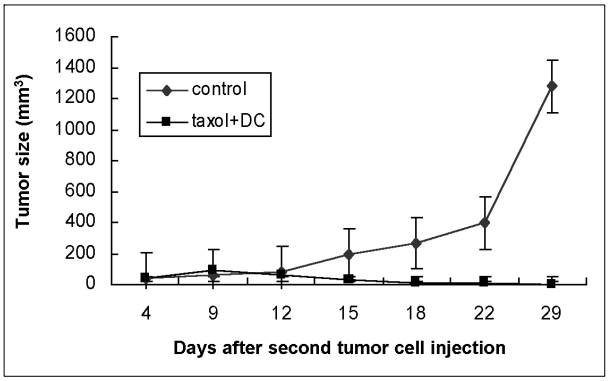


Fig. 7. Persistent antitumor memory after the combined treatment. A second challenge of the same MCA102 tumor cells to the mice, in which tumors had completely regressed in the previous experiments, was carried out. A very small-sized tumor appeared around day nine after the second injection, but it disappeared quickly thereafter. Age-matched normal mice were also injected with the same type of tumor for controls. The data represent the means ± SD of triplicate experiments.

cytotoxic T cells that were able to protect animals from a subsequent tumor challenge with the same type of tumor, suggesting that the animals had acquired long-term antitumor immunity. These results suggest that the combination therapy of paclitaxel chemotherapy together with an i.t. injection of syngeneic DCs can be a practical method for the treatment of fibrosarcoma.

DC therapy has been considered to be one of the emerging strategies for the treatment of patients with advanced cancer, especially for patients that resist conventional therapies, such as surgery, irradiation, and chemotherapy. Several different strategies using DCs in immunotherapy against tumors have been studied; these strategies are based on DCs' ability to present MHC-restricted tumor antigens. These strategies include: pulsing DCs with defined peptides or a tumor cell lysate, genetically modifying DCs to express tumor antigens and fusing DCs with tumor cells.[7-11] These manipulated DCs were then reinfused back to the recipient host. The above methods can induce CTLs directed specifically against the tumor, but they require an *ex vivo* step to expose the DCs to the tumor. The strategy used in the present study is similar to the previous strategies, but it eliminated the *ex vivo* step of loading the DCs with a tumor antigen. Instead, our DCs were injected into a tumor mass and engulfed the apoptotic bodies generated by the paclitaxel treatment directly *in vivo*. Using this method, we can eliminate the use of any surgical procedures for obtaining tumor tissues, reduce the dose of the chemotherapeutic agent to induce the apoptosis of tumor cells, and enhance direct contact of DCs to apoptotic tumor cells without migration from a remote area. Based on this hypothesis, we assumed that, in this study, the DCs we injected into the tumor recruited tumor antigens by uptaking apoptotic tumor cell bodies *in vivo*. Then, these DCs moved to the regional lymph node and activated the immune system. Therefore, our future studies should generate *in vivo* evidence that proves that DCs obtain the tumor antigen and stimulate the CTL response against the tumor after moving to the regional lymph node. Our observations also suggest that it is not necessary to identify specific tumor antigens for immunotherapy using DCs, and low-dose chemotherapy may be a sufficient method for providing antigens to DCs in the milieu of the tumor.

Tong et al., Shin et al., and Yu et al. previously reported an antitumor strategy similar to the strategy shown in the present study.[12,14,15] Tong et al. evaluated the synergistic effect of systemic cyclophosphamide chemotherapy combined with an i.t. injection of DCs for the treatment of colon adenocarcinoma and melanoma. Shin et al. tested the synergistic effect of an i.t. injection of combined vincristine and DCs for the treatment of fibrosarcoma, and Yu et al. investigated the combination of paclitaxel and DCs for the treatment of advanced stage breast cancer. We noted the following differences between our method and that of Shin's group. First, we used a different chemotherapeutic agent, paclitaxel, and this drug was injected i.p. instead of i.t. Second, the injection schedules of the antitumor drug and DCs differed. We injected DCs one day after the paclitaxel injection, whereas Shin's group injected DCs eight hours after a vincristine injection. We hypothesized that there would be more apoptotic cellular bodies available to the DCs one day after chemotherapy, and a systemic injection of the chemotherapeutic drug would generate a higher regression of the tumor. Also, we asserted that this procedure would lower the treatment burden to the host animals. Yu et al. utilized the same combined treatment but in a different tumor model

with different cycles of chemotherapy. Yu's group used 7.5 mg/kg of paclitaxel for their animal experiments but we injected a smaller amount, 5 mg/kg, in the present study. They also showed that the combination of DC administration with repeated cycles of paclitaxel and dexamethasone, which is similar to real clinical practice, resulted in the induction of antitumor response.

DCs are known to be the most potent antigen-presenting cells (APCs) of the immune system. They are capable of activating T cells in response to both new and recall antigens.[1-3] The maturation state of DCs is one of the factors that affects the capacity of DC to induce antigen-specific CTLs. We used immature DCs in our study because immature DCs have phagocytic activity, and they can effectively capture exogenous antigens. These immature cells were produced by culturing DC precursors from bone marrow in the presence of GM-CSF and IL-4 for six days. However, there is still a need to evaluate the degree to which DCs matured *in vitro* induce the antitumor effect when combined with low-dose chemotherapy. In addition, the effects of various routes of DC injection need to be evaluated. Besides these, the following issues should also be elucidated: (1) Which subset of DCs can stimulate antitumor immunity most effectively? (2) What is the potential for a combination therapy of a DC-based treatment with other therapies (such as radiotherapy or cytokine therapy)? (3) Can a steroid hormone (such as dexamethasone) used in conventional cancer treatment inhibit the anticancer immunity induced by a DC-based treatment?

In summary, a combination therapy of systemic paclitaxel chemotherapy together with an i.t. injection of DCs is a promising method for the eradication of fibrosarcoma *in vivo*. The data presented in this study and in previous studies strongly suggest that the presented strategy may overcome the drug resistance problems associated with conventional chemotherapy, and this strategy could be applied to a wide variety of tumors in clinical settings.

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