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Antitumor effect of intratumoral administration of dendritic cell combination with vincristine chemotherapy in a murine fibrosarcoma model

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Summary. A new antitumor therapeutic strategy utilizing the combined effect of chemotherapy and DC (dendritic cell)-based immunotherapy was designed, and the effect of intratumoral injections of unpulsed, immature DCs was evaluated after *in vivo* pretreatment of vincristine on tumor growth in a murine fibrosarcoma tumor model.

Vincristine exerted a much more potent apoptosis/necrosis-inducing effect on MCA-102 tumor cells than on DCs both *in vitro* and *in vivo*. Moreover, CD11c, CD40, CD80 and CD86 molecules on DCs were not downregulated after treatment with vincristine either *in vitro* or *in vivo*. The growth of tumor significantly regressed in the group which received the combined vincristine chemotherapy with intratumoral administration of DCs in contrast to the untreated group, the group treated with DCs alone, and the group treated with vincristine alone. In particular, an upregulated expression of CD40, CD80 and CD86 molecules on DCs was found in the combination treatment group. Furthermore, the number of $CD4^+$ and $CD8^+$ T cells and the staining intensity of their CD4 and CD8 surface molecules also increased after the combination treatment.

Therefore, our results indicate the feasibility of this combination therapy with vincristine chemotherapy and DC-based immunotherapy as an efficient antitumor strategy for the treatment of fibrosarcoma.

Key words: Dendritic cell-based tumor immunotherapy, Chemotherapy, Apoptotic/necrotic tumor cells, Fibrosarcoma, Tumor immunology

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Introduction

Dendritic cells (DCs) are the most effective and potent antigen-presenting cells (APCs) in the induction of primary immune response (Steinman, 1992). In most non-lymphoid tissues, DCs are present in an immature state and provide an extensive network of sentinel APCs capable of antigen capture and processing (Banchereau and Steinman, 1998). Once DCs have taken up an antigen or encountered a powerful immunological stimulus, they become activated, and subsequently migrate via lymphatics or blood vessels into the lymphoid tissues such as the spleen and lymph node where they may complete their maturation and can stimulate T cells (Hart, 1997; Banchereau and Steinman, 1998; Yoon et al., 2001). Given their central role in controlling immunity, DCs are logical targets for many clinical situations that involve T cells such as tumor, transplantation, allergy, hypersensitivity, autoimmune disease, resistance to infection, immunodeficiency, vaccination and immunotherapy (Hart, 1997; Banchereau and Steinman, 1998). Especially, DCs are considered to be attractive vectors for tumor immunotherapy because of their unique properties that include high antigen capture and presentation capacity resulting in extremely efficient induction and maintenance of tumor-specific cytotoxic T lymphocyte (CTL) responses. Stimulation with antigen-loaded DCs was shown to break tolerance to tumor-associated antigens and to induce antitumor cytotoxic immune responses *in vivo*. Since CTLs play a major role in the rejection of immunogenic tumors, the induction of stronger CTL responses has become a major goal of current cancer vaccine strategies (Melief, 1992). It has been shown recently that immature DCs can efficiently acquire antigen from apoptotic/necrotic tumor cells and induce major histocompatibility complex (MHC) class I-

restricted, antigen-specific $CD8^+$ CTLs (Albert et al., 1998a,b; Sauter et al., 2000; Candido et al., 2001; Chen et al., 2001).

Several antigen-delivery systems have been employed so far including defined peptides of known sequences (Mayordomo et al., 1995; Porgador and Gilboa, 1995; Celluzzi et al., 1996; Eggert et al., 1999), undefined acid-eluted peptides from autologous tumor (Zitvogel et al., 1996), whole tumor lysates (Fields et al., 1998), retroviral and adenoviral vectors (Song et al., 1997; Butterfield et al., 1998), tumor cell derived cDNAs, RNAs or proteins (Boczkowski et al., 1996; Brossart et al., 2001), fusion of DC with tumor cells (Gong et al., 1998), tumor peptide-pulsed DC-derived exosomes (Zitvogel et al., 1998), tumor-derived exosomes (Wolfers et al., 2001) and apoptotic/necrotic tumor cell-phagocytosed DCs (Nouri-Shirazi et al., 2000; Chen et al., 2001). Hence, numerous attempts to optimize delivery of tumor antigens to DCs, as well as routes and schedules of administration to cancer patients, are currently under way. However, each of these methods has drawbacks (Sogn, 1998). Foremost, the use of MHC class I-binding peptides, and viral vectors encoding the cDNA for defined tumor peptides is associated with MHC restriction and does not tend to effectively elicit MHC class II-restricted $CD4^+$ T cell-mediated immune response and thus the induced immune responses tend to be limited only to $CD8^+$ T cells (Stuhler et al., 1999). Furthermore, the process of identifying MHC class I-binding tumor peptides is labor-intensive and time consuming, and only a small number of human tumor peptides have been identified (Kawakami et al., 1994; Tsai et al., 1997). The other major strategies to deliver undefined tumor antigens including tumor cell-derived cDNAs, RNAs, peptides or proteins, and apoptotic/necrotic tumor cells to DCs are commonly associated with a problem that these approaches require enough tumor cells isolated from cancer patients for *in vitro* pulse of tumor antigens to DCs.

Thus, a novel efficient strategy to overcome this potential limitation in DC-based tumor vaccine development was designed in the present study. Since chemotherapy has been the most widely used method in the treatment of tumors and it induces apoptotic/necrotic tumor cell death, it is conceivable that DCs may offer an efficient means for triggering anti-tumor immune responses *in situ* within tumor tissues if DCs are injected into tumor where apoptotic/necrotic tumor cells were induced by optimal chemotherapeutic agents. In this aspect, the first object for the present study was to identify optimal chemotherapeutic agents, to which tumor cells should be sensitive so that apoptotic/necrotic cell death can be induced and at the same time, to which DCs should be relatively resistant, when the same dose of chemotherapeutic agents were treated to tumor cells and DCs. To search for such optimal chemotherapeutic agents satisfying these criteria, we first assessed *in vitro* cytotoxic effects of various antineoplastic agents such as an antimetabolite (5-fluorouracil), alkylating agents (cyclophosphamide, ifosfamide), antimitotic drugs (vinblastine and vincristine), antibiotics (doxorubicin and bleomycin), an epipodophyllotoxin (etoposide) and a platinum compound (cisplatin) on MCA-102 tumor cells. Then the result was applied to *in vivo* tests. Taking into consideration of the overall *in vitro* and *in vivo* results, vincristine was selected as an optimal chemotherapeutic agent for the combined chemotherapy and DC-based immunotherapy in the present study.

Therefore, the present study was performed to evaluate the effect of intratumoral administration of tumor antigen-unpulsed, immature DCs combined with vincristine chemotherapy on the growth of the murine MCA-102 fibrosarcoma cells *in vivo* and thus to define an efficient antitumor DC vaccine strategy.

Materials and methods

Animals

Female C57BL/6 ($H\text{-}2K^b$) mice were purchased from Dae Han Bio Link (Seoul, Korea). All animals were housed at 4 - 5 mice per cage and maintained under a 12-hr light/dark cycle at a temperature of 20-22 °C in a pathogen-free facility. Food and water was available *ad libitum*. Mice were allowed to adjust to their environment for 1 week and used at 6-8 weeks of age for DC isolation and at 8-10 weeks of age for tumor inoculation. Animal care and all experimental procedures were conducted in accordance with the "Guide for Animal Experiments edited by the Korean Academy of Medical Sciences".

Chemotherapeutic agents, antibodies and cytokines

Chemotherapeutic agents including bleomycin, cisplatin, cyclophosphamide, doxorubicin, ifosfamide, etoposide, 5-fluorouracil (5-FU), vinblastine and vincristine were all purchased from Sigma (St. Louis, MO, USA). Monoclonal antibodies including rat anti-mouse MHC class II^b (Ia^b), CD3, CD4, CD8, CD11c (N418), CD19, CD40, CD80 and CD86 antibodies were all purchased from PharMingen (San Diego, CA, USA). The recombinant mouse interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Peprotech (Rocky Hill, NJ, USA).

Tumor cell culture

MCA-102 is a murine fibrosarcoma cell line of C57BL/6 mouse origin. This cell line was maintained in the complete medium that is RPMI-1640 (GIBCO-BRL, Rockville, MD, USA) medium plus 10% heat-inactivated fetal bovine serum (FBS, GIBCO-BRL), 100 U/ml penicillin (GIBCO-BRL), 100 μg/ml streptomycin (GIBCO-BRL) in a 5% CO_2 incubator at 37 °C to a subconfluent state in 100 mm dishes or 96-well plates

(Nunc, Denmark). After reaching subconfluence, cells were rinsed twice with phosphate-buffered saline (PBS), or RPMI-1640. For detachment, cells grown to confluence were treated with 0.05% trypsin (GIBCO-BRL) and 0.53 mM ethylenediaminetetraacetic acid (EDTA, GIBCO-BRL). For *in vivo* inoculation, tumor cells were washed three times with brief incubations in Hanks Basic Salt Solution (HBSS, GIBCO-BRL).

Murine DC culture

Bone marrow cells were isolated from the femur and tibia of C57BL/6 mice and depleted of erythrocytes by ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA [pH 7.2-7.4]). Remaining bone marrow cells were washed twice with HBSS and then resuspended in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 50 μM 2-mercaptoethanol, 20 ng/ml murine rGM-CSF and 10 ng/ml murine rIL-4 at 37 °C in a humidified 5% CO_2 atmosphere. On day 3, non-adherent granulocytes and B and T lymphocytes were gently removed, and fresh media were added. On day 6 of culture, released, immature, non-adherent cells with the typical morphological features of DCs were harvested and used for MTT assay, cell death assay and flow cytometric analysis, and for *in vivo* intratumoral injection. Cultures revealed a purity of DC exceeding 70% as detected by a flow cytometric analysis based on the expression of CD11c, MHC class II molecules, CD40, CD80 (B7.1) and CD86 (B7.2). For *in vivo* administration, DCs were washed three times with brief incubations in HBSS.

MTT assay

The number of viable cells at the end of the culture was quantified by the MTT assay (Mosmann, 1983). The tumor cells and DCs in their own complete media ($1x10^4$/ml) were incubated in 96-well flat-bottomed microplates (Nunc, Roskilde, Denmark) containing serial dilutions of the chemotherapeutic drugs in a total volume of 200 μl/well and incubated for 48 hr. The concentrations of the employed dilutions of cisplatin, cyclophosphamide, doxorubicin, etoposide, 5-FU and vinblastine were 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 μM. The concentrations of the employed dilutions of bleomycin, ifosfamide and vincristine were 0.001, 0.005, 0.01, 0.05 and 0.1 μg/ml. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) was dissolved in Dulbecco's phosphate-buffered saline at 5 mg/ml. Following exposure to the chemotherapeutic drugs, 1/10 diluted MTT solution (0.5 mg/ml) was added to the assay wells (100 μl per 200 μl medium). The plates were incubated at 37 °C for 4 hr and the dark blue crystals of MTT-formazan were observed at the bottom of the wells. The culture plates were centrifuged at 150 g for 10 min, and the supernatant was discarded. The formazan precipitate was dissolved with dimethylsulphoxide (DMSO), and was mixed in a plate shaker for 20 min. The amount of converted MTT was quantified in an ELISA reader (Bio-Tek Instruments, Inc., Winooski, Vermont, USA) using 570 nm test and 650 nm reference wavelengths. The results were expressed as the mean ± SD of absorbance. Each experiment on each chemotherapeutic agent was performed in six preparations and repeated four times independently.

Measurement of apoptotic/necrotic cell death by fluorescence microscopy

Nuclear morphology was analyzed with fluorescent DNA-binding dyes, Hoechst 33258 (H33258, Sigma), to assess the rate of occurrence of apoptosis among MCA-102 cells and DCs. The tumor cells and DCs were collected by centrifugation for 5 min at 1000 rpm 12 and 20 hr after vincristine (0.2 μg/ml) treatment. They were fixed in 4% paraformaldehyde for 10 min and stained with H33258 (1 μg/ml) for 20 min at 37 °C. Cell pellets were resuspended in a small volume (100 μl) of PBS containing 1 μg/ml H33258. An aliquot (20 μl) was placed on the slide glass and covered with a glass cover slip. For morphological detection of apoptotic cells, individual nuclei were visualized to detect the characteristic condensed coalesced chromatin pattern of apoptotic cells. Control cells and cells exposed to the chemotherapeutic agents were simultaneously stained with Annexin V (Pharmingen) and propidium iodide (PI, Sigma) to differentiate apoptotic and necrotic cell death phenotypes. Cells were rinsed with phosphate-buffered saline (PBS) and incubated in staining solution consisting of HEPES buffer and Annexin V fluorescein labeling reagent, and PI for 15 min in the dark. Stained cells were examined with a Zeiss Axioskop fluorescence microscope (Oberkochen, Germany) fitted with a filter combination that allowed green and red fluorescing cells.

Flow cytometric analysis of DCs after treatment of vincristine

For phenotypic analysis, DCs from the bone marrow culture and DCs that were treated with different concentrations of vincristine were stained with a panel of antibodies and then quantified by flow cytometry. The antibodies used were rat anti-mouse Ia^b, CD40 and CD86 and hamster anti-mouse CD11c (N418) and CD80 antibodies. Briefly, DCs were incubated with each of the above antibodies (5 μg/ml) on ice for 30 min. After 3 washes with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rat or anti-hamster IgG antibodies (Vector Laboratories Inc., Burlingame, CA, USA) on ice for another 30 min. After 3 washes with PBS, cells were then analyzed by a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). Isotype-matched monoclonal

antibodies were used as controls.

In vivo treatment of tumor, drug and DCs

To examine whether vincristine induces apoptotic/necrotic tumor cell death *in vivo*, sixteen C57BL/6 mice received 100 μl of 1x10^5 viable MCA-102 tumor cells s.c. in the right flank on day 0. On day 9, when the tumor size reached about 3 - 5 mm in diameter, the mice were intratumorally injected with vincristine (0.5 mg/kg) and the tumor tissues were analyzed 12 hr later.

For evaluation of the antitumor effect of the *in vivo* combination treatment with a chemotherapeutic agent and DCs, mice were injected with DCs into tumor tissues 8 hr after vincristine treatment. A total of sixty experimental animals were divided into the untreated group, the group treated with DCs alone, and the group treated with vincristine alone, and the group receiving combination treatment with vincristine and DCs. Each group consisted of fifteen C57BL/6 mice. They received 100 μl of 1x10^5 viable MCA-102 tumor cells s.c. in the right flank on day 0. On day 9, when the tumor size reached at about 3-5 mm in diameter, mice were intratumorally injected with vincristine (0.5 mg/kg) and 100 μl of 2x10^6 DCs, and then the same treatment was repeated on day 13, 17, and 21. In this experimental group, syngeneic DCs derived from C57BL/6 mice were always administered 8 hr after vincristine treatment. Control mice received HBSS. The size of the tumor was assessed in a blind, coded fashion on day 9, 13, 17, 21 and 25 by measuring the largest perpendicular diameters with calipers. Data were presented as the average tumor size ± SD.

Tissue preparation and histological analysis

For the tissue preparation, mice were anesthetized with a single intraperitoneal injection of sodium pentobarbital (5 mg/kg body weight). Tumor and the surrounding rim of normal skin and underlying connective tissues were excised and submitted for histological processing. For frozen section, the tumor tissues were rapidly frozen in isopentane cooled with liquid nitrogen. Frozen sections (5 μm thick) were cut on a Reichert cryostat and placed on 3-aminopropyl-triethoxysilane-coated slides. After being dried, the cryosections were fixed in cold acetone for 10 min at -20 °C. For paraffin section, the tissues were fixed in 4% paraformaldehyde and neutral-buffered formalin. Then, the specimens were dehydrated in graded ethanol, embedded in paraffin, and cut into 4-μm-thick sections on a Reichert microtome. The paraffin sections were mounted on 3-aminopropyltriethoxysilane-coated slides overnight at 40 °C. The slides were stained with H&E and histological analysis was performed.

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 being washed with phosphate-buffered saline, tissue slides were fixed in 3% paraformaldehyde (20 min at room temperature) followed by permeabilization with 0.2% triton X-100 (15 min at room temperature). The TUNEL assay was conducted according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Boehringer Mannheim, Mannheim, Germany). The preparations were then rinsed with Tris-buffered saline and incubated (12 min at room temperature) with 5-bromo-4-chloro-indolyl phosphate and Nitro Blue Tetrazolium liquid substrate system (Sigma).

Immunohistochemistry

Immunostaining was undertaken using the streptavidin-biotin complex (ABC) method. In brief, sections were incubated for 10 min in a solution of phosphate-buffered saline (PBS) containing 0.3% H_2O_2 to eliminate endogenous peroxidases. After a wash in PBS, the sections were incubated with a bovine serum albumin solution (Sigma, 10 mg/ml in PBS). The excess solution was shaken off and the sections were incubated for 24 hr at 4 °C with the primary monoclonal antibodies (mAbs). Following incubation with the primary antibodies, the tissue preparations were washed 3 times, each for 5 min, with PBS and incubated with biotinylated anti-rat or anti-hamster antibody (Vector Laboratories Inc.). Then the sections were rinsed in PBS, and incubated for 60 min at room temperature with an ABC reagent (Vectastain Elite kit, Vector Laboratories Inc.) according to the manufacturer's instructions. After a PBS rinse, the sections were developed with a 0.05% 3-3'-diaminobenzidine-H_2O_2-medium under microscopical control at room temperature to visualize the activity of peroxidase. Afterwards, the sections were mounted in a xylene-based mounting medium (Permount).

Two-color immunohistochemical analysis was performed in order for the precise identification the types of cell which express CD40, CD80 and CD86 costimulatory molecules according to the procedure of our previous study (Yoon et al., 2001). In brief, the cryosections were fixed in cold acetone for 10 min, and then in formol calcium solution for 2 min after rehydration in PBS. After washing in PBS and incubation with 2% BSA solution for 20 min, the sections were incubated with the first primary mAbs for 16-18 hr at 4 °C. Following incubation with the primary mAbs, the sections were washed with PBS and incubated with biotinylated secondary antibodies (Jackson Immunoresearch Labs. and Vector Labs.). Then the sections were further fixed with 1% glutaraldehyde in PBS for 30 sec. After the sections were incubated with an ABC reagent (Vectastain Elite kit), they were developed with a 0.05% 3-3'-diaminobenzidine-H_2O_2-medium. Thereafter, the sections were incubated with the second primary mAbs, and then with alkaline

phosphatase-labeled secondary antibodies. Finally, the labeled cells were colored blue by the alkaline phosphatase substrate kit (Vector Blue, Vector Labs.). The sections were mounted in Vectashield (Vector Labs.).

Controls for the staining procedure included the following: (1) omission of primary antibodies from the reaction sequence as well as their replacement with normal rat or hamster IgG (Vector Laboratories Inc.); and (2) omission of the secondary antibody as well as ABC solution from the reaction sequence.

Immunostained sections were interpreted and evaluated by simultaneous viewing of the slides by two observers in a blind fashion, using a double-headed microscope. The slides were observed and photographed under an Olympus BX 50 microscope.

Statistical analysis.

Data are expressed as means ± standard deviation (SD) for each condition. For comparisons of two treatment groups, a Student's non-paired *t* test was performed. For comparisons of multiple treatment groups, a one-way analysis of variance (ANOVA) followed by a Scheffe's post hoc test was performed using tumor size measurements taken at each time point. Statistical significance was achieved when $p<0.05$.

Results

The in vitro cytotoxic effect of vincristine on tumor cells was much more potent than the effect on DCs

Vincristine (above 0.05 μg/ml) exhibited a significant cytotoxic effect on MCA-102 tumor cells when the viability of MCA-102 tumor cells was assessed by MTT assay (Fig. 1A). Although 5-FU also showed a significant cytotoxic effect (data not shown), other chemotherapeutic agents (bleomycin, cisplatin, cyclophosphamide, doxorubicin, etoposide and vinblastine) exhibited no remarkable cytotoxic effect at the concentrations employed on MCA-102 tumor cells. In particular, the cytotoxic effect of vincristine on MCA-102 tumor cells was much more potent than the effect on DCs under the same conditions (Fig. 1A,B).

Significant apoptotic/necrotic morphological changes were observed by Hoechst stain in the MCA-102 tumor cells 12 and 20 hr after vincristine (0.2 μg/ml) treatment (Fig. 2). The induction of apoptosis was further confirmed using Annexin V-FITC Apoptosis Detection kit. As shown in Fig. 3, a significant number of apoptotic cells appeared in the MCA-102 tumor cells 12 and 20 hr after treatment of vincristine (0.2 μg/ml). These results indicate that these tumor cells underwent apoptosis and necrosis after vincristine treatment. In contrast to the tumor cells, however, only a few DCs displayed apoptotic/necrotic morphological changes at 12 hr after treatment of 0.2 μg/ml vincristine (Fig. 4).

Vincristine did not affect the phenotype of DCs in vitro

The immature DCs used in this study showed a typical morphological characteristic of DCs with numerous dendrites by immunofluorescence analysis, and a significant expression of MHC class II (Ia^b) antigen, CD11c, CD40, CD80 and CD86 molecules (Fig. 5) but no expression of CD3 (T-cell marker) and CD19 (B-cell marker).

The expression level of MHC class II (Ia^b) antigen, CD11c, CD40, CD80 and CD86 molecules on vincristine-treated (0.05, 0.1 and 0.2 μg/ml) DCs remained mostly unchanged in comparison with those

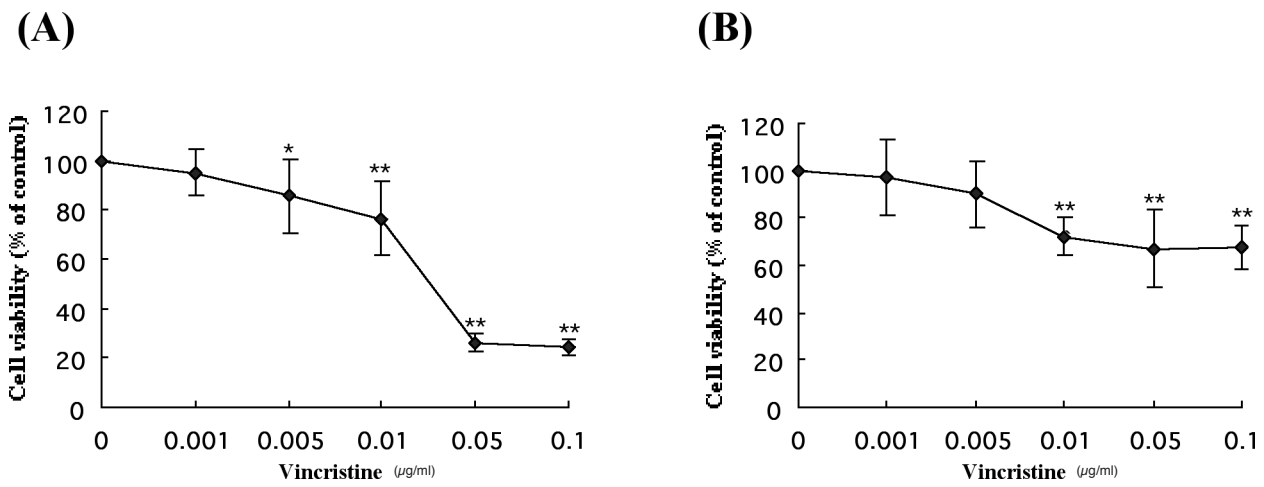

Fig. 1. The viability of MCA-102 fibrosarcoma cells **(A)** and DCs **(B)** 48 hr after vincristine treatment assessed by MTT assay. Vincristine (above 0.05 μg/ml) exhibits a strong cytotoxic effect on MCA-102 tumor cells and the cytotoxic effect of vincristine on tumor cells is much more potent than the effect on DCs under the same conditions. Data are expressed as the mean ± SD of six preparations of cultures, and are representative of four separate experiments. *: $p<0.05$ and **: $p<0.01$ compared with the corresponding control value, as determined by Student's non-paired t test.

molecules on control DCs by flow cytometric analysis, although vincristine above 0.1 μg/ml resulted in a down-regulated expression of Ia antigen on DCs (Fig. 5).

Vincristine induced tumor cell death but did not exhibit significant suppressive effect on costimulatory molecule expression and DC number in vivo

To examine whether vincristine, which exhibited tumor cell death *in vitro* was capable of induction of tumor cell death also *in vivo*, tumor tissue sections stained with H&E were analyzed at 12 hr after intratumoral injection of 0.5 mg/kg vincristine. The tumor tissues from vincristine-treated mice exhibited numerous apoptotic/necrotic tumor cells by H&E stain (Fig. 6A,B). Apoptosis and necrosis of MCA-102 tumor cells could be easily identified by the cellular morphology. TUNEL stain also revealed a definite apoptotic cell death-inducing effect of vincristine (Fig.

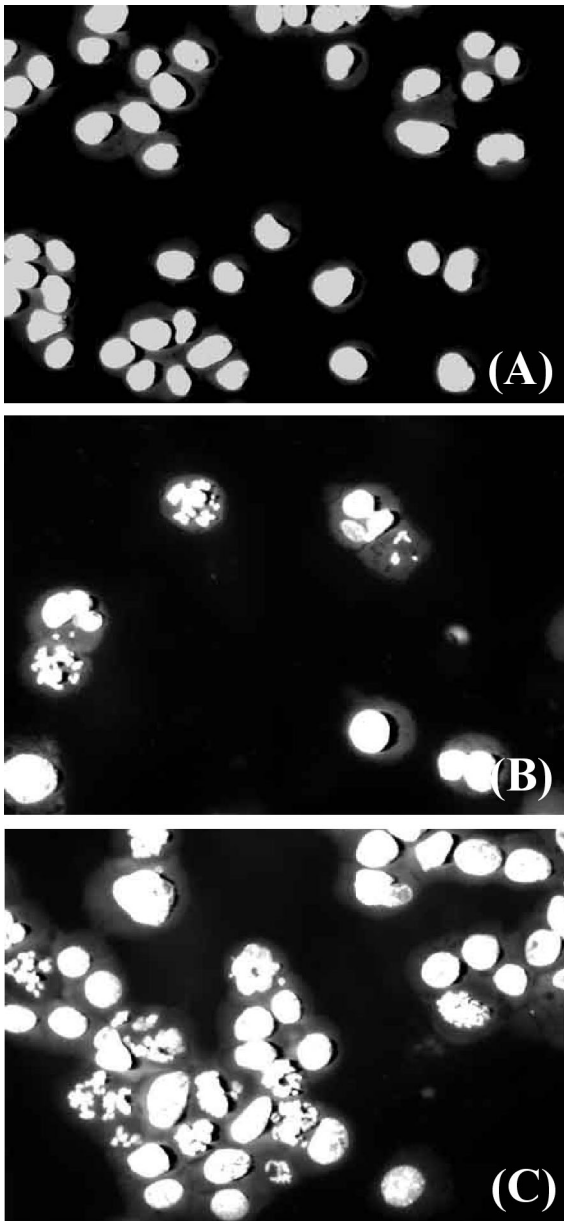

Fig. 2. Apoptosis/necrosis of MCA-102 fibrosarcoma cells induced by vincristine treatment *in vitro*. In contrast to the control group **(A)**, many MCA-102 cells exhibiting apoptotic/necrotic changes are observed in the groups 12 hr **(B)** and 20 hr **(C)** after 0.2 μg/ml vincristine treatment. Hoechst stain. x 200

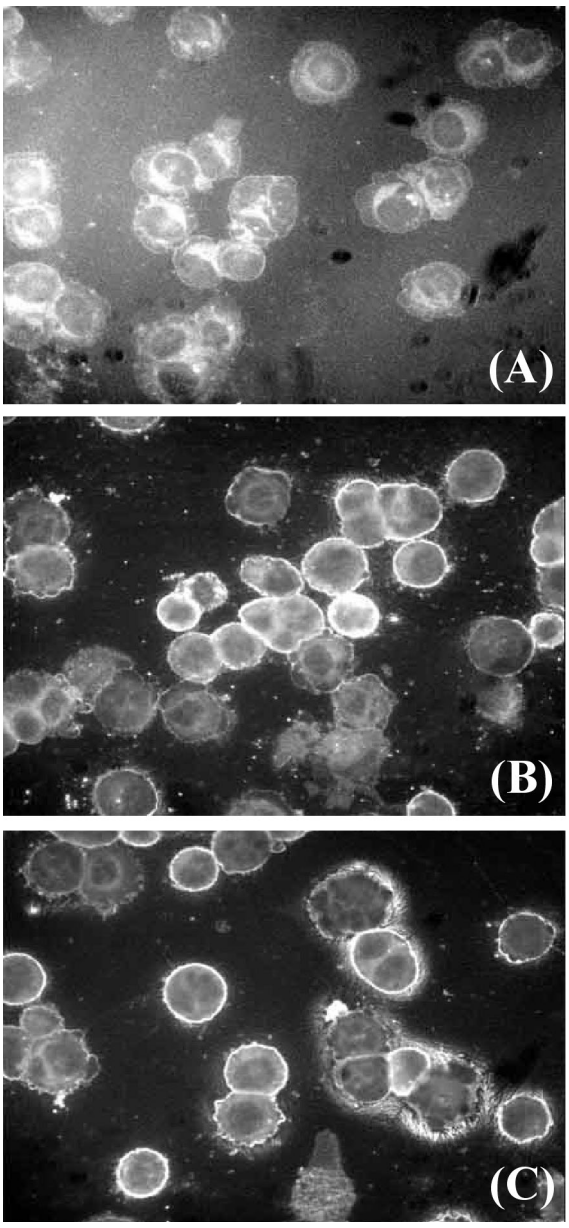

Fig. 3. Apoptosis of MCA-102 fibrosarcoma cells assessed by annexin V staining after vincristine treatment. In contrast to the control cells **(A)**, many MCA-102 cells 12 hr **(B)** and 20 hr **(C)** after 0.2 μg/ml vincristine treatment clearly show intense membrane staining. x 400

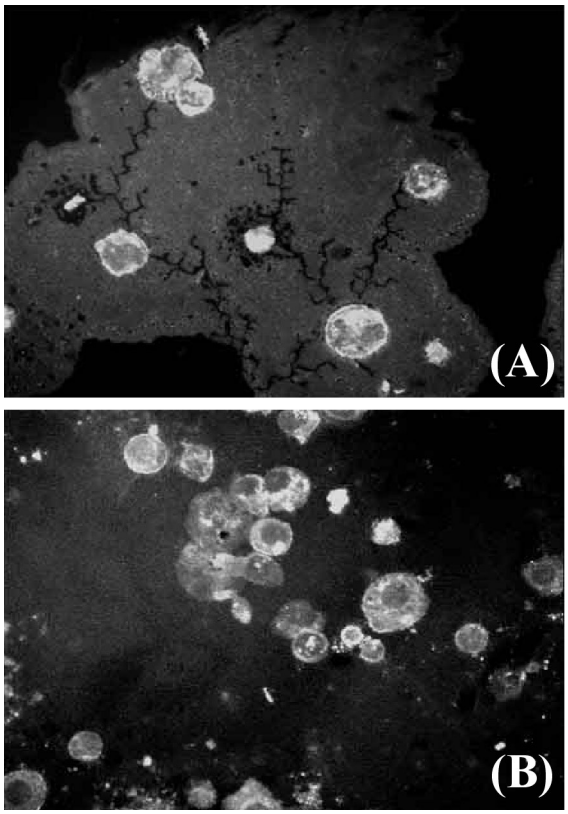

Fig. 4. Apoptotic assay of DCs after vincristine treatment by annexin V staining. Both control cells **(A)** and DCs **(B)** 12 hr after 0.2 μg/ml vincristine treatment do not display positive immunoreactivity in the cell membrane by fluorescence microscopy, indicating apoptosis of DCs was not induced by vincristine treatment. x 400

6C,D).

Vincristine (0.5 mg/kg) did not show a significant alteration in the expression of CD40, CD80 and CD86 costimulatory molecules (Fig. 7). Vincristine (0.5 mg/kg) did not display either a suppressive effect on CD11c expression and DC number (data not shown).

Combined vincristine chemotherapy with intratumoral administration of DCs induced upregulated expression of costimulatory molecules and an increase in the number of CD4$^+$and CD8$^+$T cells

Double-staining revealed that most of the cells which express CD40, CD80 and CD86 costimulatory molecules in the tumor tissue were the N418$^+$ or Ia$^+$ DCs (Fig. 8). Noticeably, an upregulated expression of CD40, CD80 and CD86 molecules on DCs was found in the combination treatment group when compared to the other groups (Fig. 7). CD4$^+$ and CD8$^+$ T cells did not exhibit significant alterations in their cell number and

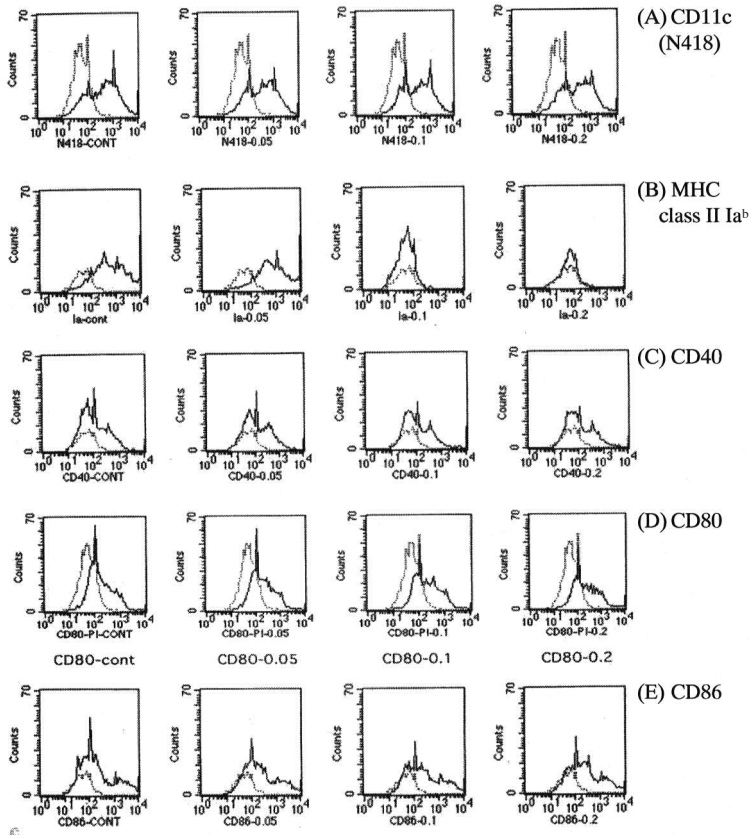

Fig. 5. Phenotypic analysis of DCs by vincristine treatment. The expression of Ia^b, CD11c, CD40, CD80 and CD86 was analyzed in bone marrow-derived murine DCs after treatment with different concentrations of vincristine (0.05, 0.1 and 0.2 μg/ml). Control cells (CONT) were stained with isotype-matched monoclonal antibodies. Control DCs display a significant expression of MHC class II (Ia^b) antigen, CD11c, CD40, CD80 and CD86 molecules. The expression level of MHC class II (Ia^b) antigen, CD11c, CD40, CD80 and CD86 molecules on vincristine-treated DCs remained mostly unchanged in comparison with those molecules on control DCs, although vincristine above 0.1 μg/ml shows in a downregulated expression of Ia^b antigen on DCs.

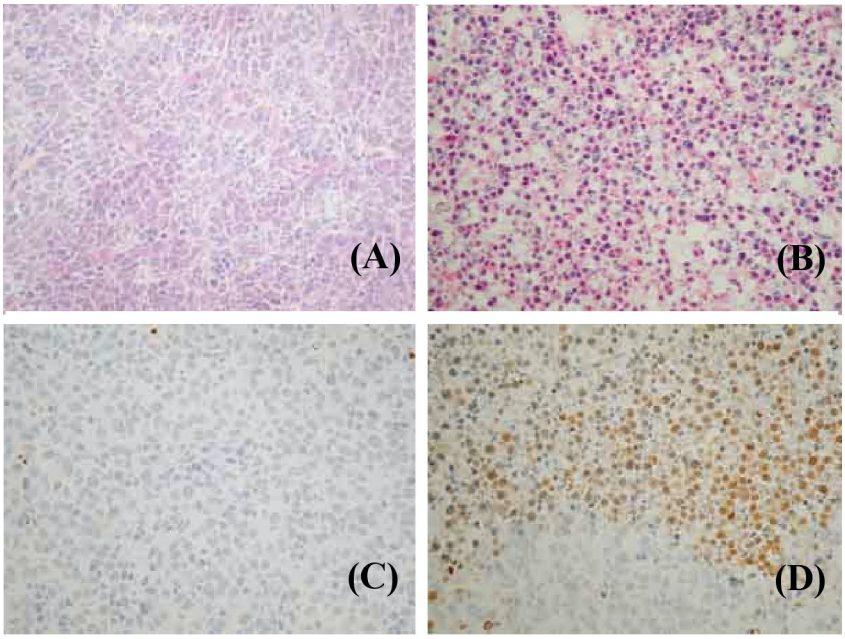

Fig. 6. Apoptosis of MCA-102 tumor cells in the tumor tissues after vincristine treatment assayed by H&E stain **(A, B)** and TUNEL stain **(C, D)**. Whereas there are few apoptotic/necrotic cells in the control group **(A, C)**, numerous apoptotic/necrotic tumor cells are observed in mice 8 hr after vincristine (0.5 mg/kg) injection **(B, D)**. x 200

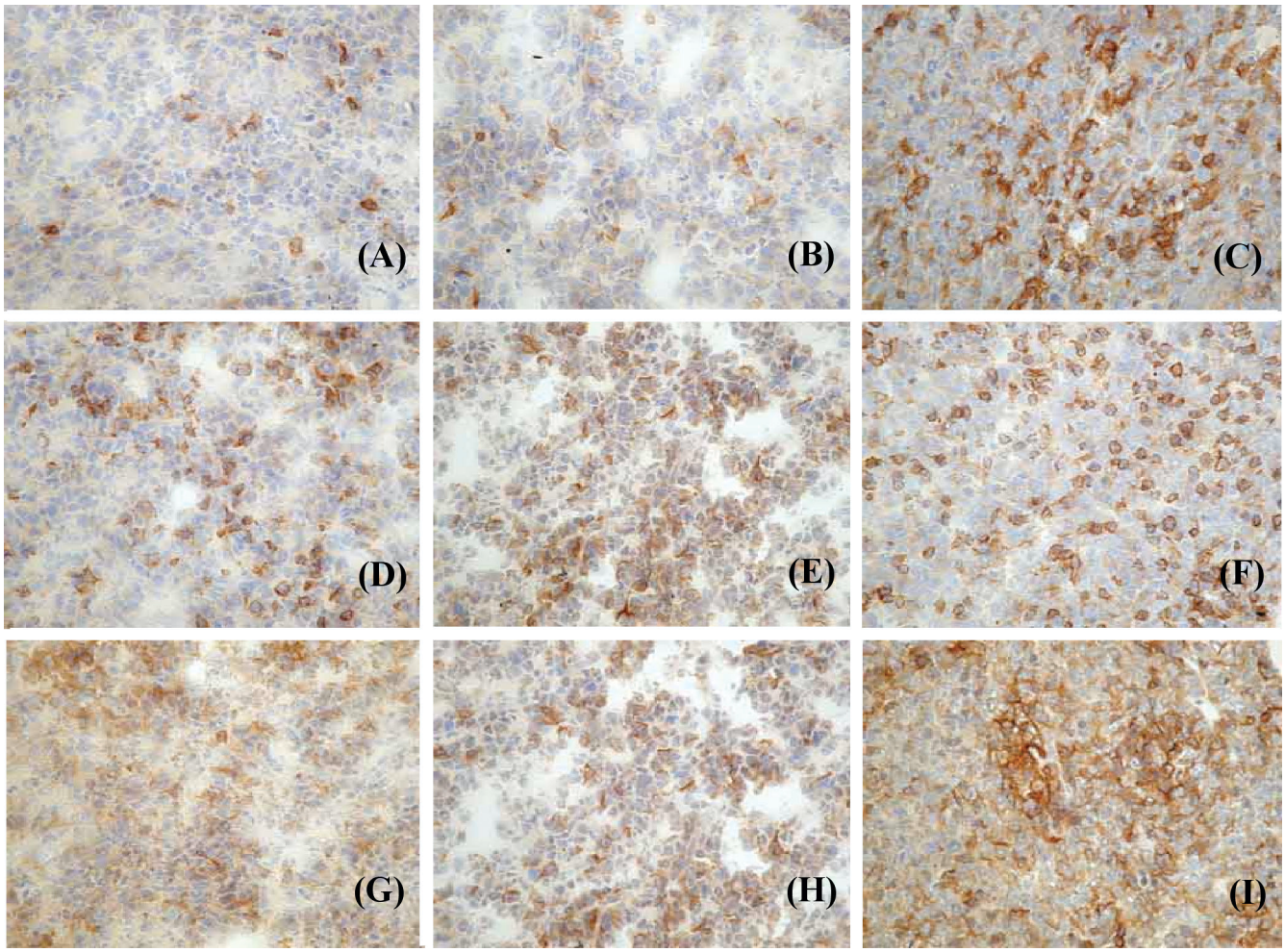

Fig. 7. Immunostaining for costimulatory molecules, CD40 **(A-C)**, CD80 **(D-F)** and CD86 **(G-I)** in the tumor tissues. Tumor tissues from the untreated control group **(A, D, G)**, the group treated with 0.5 mg/kg vincristine **(B, E, H)**, and in the combination treatment group **(C, F, I)** were excised 4 days after the second combination treatment of vincristine and DCs. Cryocut sections were immunohistochemically stained. The group treated with vincristine shows no significant alteration in CD40 **(B)**, CD80 **(E)** and CD86 **(H)** expression compared with the corresponding control group **(A, D, G)**. An intense CD40 **(C)**, CD80 **(F)** and CD86 **(I)** staining is noted in the combination treatment group. x 200

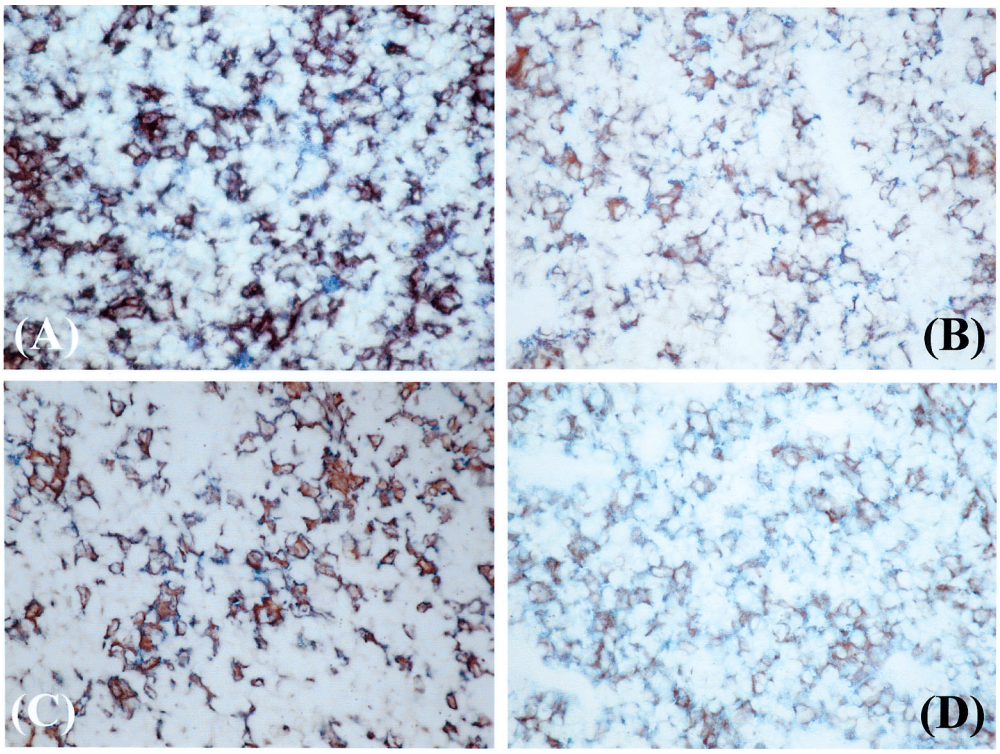

Fig. 8. Two-color double-staining of **(A)** Ia$^+$ cells (brown color) and N418$^+$ cells (blue color), **(B)** CD40$^+$ cells (brown color) and N418$^+$ cells (blue color), **(C)** Ia$^+$cells (brown color) and CD80$^+$cells (blue color) and **(D)** N418$^+$ cells (brown color) and CD86$^+$ cells (blue color) in the tumor tissues 4 days after the second combination treatment of vincristine and DCs. Double-staining shows that most of the cells which express CD40, CD80 and CD86 costimulatory molecules in the tumor tissue are the N418$^+$ or Ia$^+$ DCs. x 400

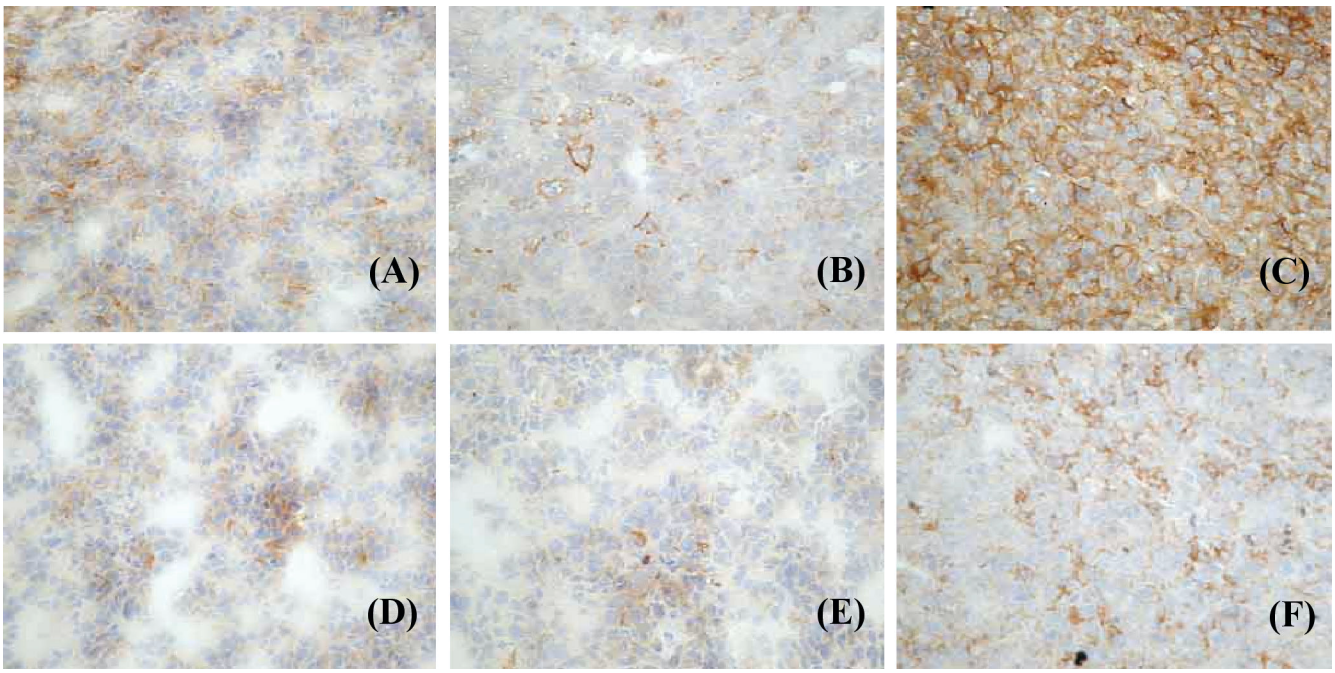

Fig. 9. Immunostaining for CD4 **(A-C)** and CD8 **(D-F)** molecules in the tumor tissues. Tumor tissues from the untreated control group **(A, D)**, the group treated with 0.5 mg/kg vincristine **(B, E)**, and in the combination treatment group **(C, F)** were excised 24 hr after the third combination treatment of vincristine and DCs. Cryocut sections were immunohistochemically stained. The group treated with vincristine shows no significant alteration in CD4 **(B)** and CD8 **(E)** expression compared with the corresponding control group **(A, D)**. An increase in the number of positively stained cells and in their staining intensity is noted in the combination treatment group **(C, F)**. x 200

surface molecule expression in the vincristine-treated group (Fig. 9). Importantly, the number of $CD4^+$ and $CD8^+$ T cells was increased and the staining intensity of CD4 and CD8 molecules was also increased after the combination treatment with vincristine and DCs (Fig. 9).

The growth of tumor significantly regressed in the combination treatment group in contrast to the other groups

To determine whether the combined vincristine chemotherapy with intratumoral administration of DCs is capable of induction of efficient antitumor immunity, DCs were injected into tumor tissues 8 hr after vincristine (0.5 mg/kg) treatment. As shown in Fig. 10, tumor growth was profoundly suppressed in the combination treatment group compared with that in the untreated group, the group treated with DCs alone, and the group treated with vincristine alone. On day 16 of vincristine and DC treatment (25 days after initial tumor cell injection), tumors had grown in the untreated control mice to 13.94±4.01 mm from the initial size of 3.71±1.99 mm, in the DC-alone treated group to 14.00±3.62 mm ($p>0.05$ relative to the untreated group) from the initial size of 4.85±0.97 mm, and in the vincristine-alone treated group to 6.85±3.03 mm ($p>0.05$ relative to the untreated group) from the initial size of 4.60±0.57 mm (Fig. 10). Remarkably, at this time, tumors had significantly regressed in the combination treatment group to 2.19±2.19 mm ($p<0.05$ relative to the untreated group and the DC-alone group) from the initial size of 4.73±0.86 mm (Fig. 10).

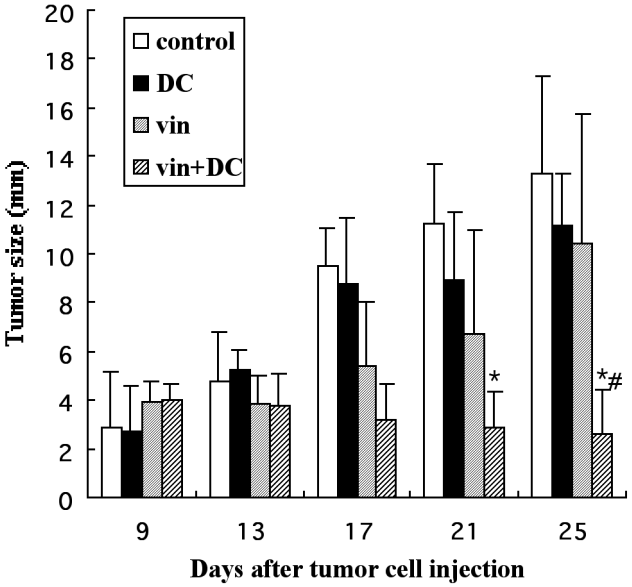

Fig. 10. The effect of combined vincristine chemotherapy with intratumoral injection of DCs on the growth of MCA-102 fibrosarcoma cells. C57BL/6 mice were received $1x10^5$ viable MCA-102 tumor cells s.c. in the right flank on day 0. On day 9, when the tumor size reached at about 3 - 5 mm in diameter, mice were intratumorally injected with vincristine (0.5 mg/kg) and 8 hr later, $2x10^6$ DCs. Then the same treatment was repeated on day 13, 17, and 21. Control mice received HBSS. Each group consisted of 15 mice. The size of the tumor was assessed on day 9, 13, 17, 21 and 25, and recorded as tumor size (in mm).

The difference between tumor sizes in the untreated control group and the combination treatment group was statistically significant at 12 and 16 days after the first combination treatment (4 days after the third and fourth combination treatment, respectively). The difference between tumor sizes in the DC-alone treated group and the combination treatment group was also statistically significant at 16 days after the first combination treatment (4 days after the fourth combination treatment). Data are expressed as the average tumor size±SD, and are representative of two independent experiments. *: $p<0.05$ compared with the corresponding untreated control value, and #: $p<0.05$ compared with the DC-alone treated group, as determined by a one-way analysis of variance (ANOVA) followed by a Scheffe's post hoc test.

The difference between tumor sizes in the untreated control group and the combination treatment group was statistically significant at 12 days after the first combination treatment (4 days after the third combination treatment) ($p<0.05$), and at 16 days after the first combination treatment (4 days after the fourth combination treatment) ($p<0.05$). The difference between tumor sizes in the DC-alone treated group and the combination treatment group was also statistically significant at 16 days after the first combination treatment (4 days after the fourth combination treatment) ($p<0.05$).

There was no significant difference in tumor size between the vincristine-alone treated group and the combination treatment group, between the untreated control group and the DC-alone treated group, between the untreated control group and the vincristine-alone treated group, and between the DC-alone treated group and the vincristine-alone treated group at any time.

Discussion

The results of the present study demonstrated that the combined vincristine chemotherapy with intratumoral administration of unpulsed, immature DCs markedly inhibited the growth of the established tumor, and thus this novel method can serve as an efficient antitumor therapeutic strategy. Although numerous strategies have been developed to employ DCs for tumor immunotherapy, most of them are basically related with the technique for *ex vivo* manipulation of DCs to deliver tumor antigens (Brossart et al., 2001; Gunzer and Grabbe 2001; Gunzer et al., 2001; Nencioni and Brossart, 2001). The present study has a major advantage compared to previous approaches in that this strategy does not require steps for the *ex vivo* loading of tumor antigens into DCs. Remarkably, however, during our manuscript preparation, Tong et al. (2001) reported essentially much the same antitumor strategy and results as the present study. They evaluated a synergy effect of conventional chemotherapy together with intratumoral injection of syngeneic bone marrow-derived DCs for the treatment of preexisting tumors, using murine CT26 colon adenocarcinoma cells derived from BALB/c mice

and B16 melanoma derived from C57BL/6 mice (Tong et al., 2001). In accordance with the results of the present study, they demonstrated that the addition of chemotherapy combined with local intratumoral injection of DCs led to a significant regression of established tumors, although the present study used local chemotherapy while they used systemic chemotherapy to induce tumor cell death (Tong et al., 2001). Therefore, our data support the results by Tong et al. (2001) and these findings indicate the feasibility of combination therapy with the intratumoral injections of DCs and chemotherapy for the treatment of cancer.

A number of studies have shown that dead (apoptotic/necrotic) cells and their "debris" can be a source of exogenous antigens for cross-presentation (Fonteneau et al., 2001). DCs can acquire antigen(s) by uptake of apoptotic tumor cells or bodies, and necrotic cells or their contents released in the extracellular environment, which in turn can induce DC maturation and lead to elicitation of MHC class I-restricted CTLs and antitumor immunity (Albert et al., 1998a,b; Henry et al., 1998, 1999; Rovere et al., 1998, 1999; Berard et al., 2000; Hoffmann et al., 2000; Nouri-Shirazi et al., 2000; Sauter et al., 2000; Bhardwaj, 2001; Larsson et al., 2001). Such a process may play an important physiological role in the acquisition of foreign antigens *in vivo*, including those derived from tumors, virally infected and normal tissues, as well as organ transplants (Bhardwaj, 2001; Candido et al., 2001; Fonteneau et al., 2001; Larsson et al., 2001). Thus, these results indicate that apoptotic tumor cells may be a good source of tumor antigens for presentation to DCs.

In general, antigen processing is maximal in immature DCs. Thus immature DCs were used for intratumoral injection in the present study. Chen et al. (2001) recently demonstrated that the immature DCs that had phagocytosed a mixture of apoptotic/necrotic tumor cells generated efficient antitumor immunity. Candido et al. (2001) suggested that the balance between the levels of apoptotic *versus* necrotic cells within a tumor mass influences the capacity of DCs to trigger an effective immune response *in situ*, which may lead to a good *versus* a poor prognosis.

As a result of the *in vitro* tests, vincristine was determined to be an optimal chemotherapeutic agents for the *in vivo* tests since vincristine was found to exert a significant apoptosis/necrosis-inducing effect on MCA-102 tumor cells but not to exhibit a significant apoptosis/necrosis-inducing effect on DCs. In addition, MHC class II (Ia^b) antigen, CD11c, CD40, CD80 and CD86 molecules on DCs did not display a significant downregulated expression after treatment with vincristine. By the *in vivo* tests, it was also demonstrated that not only MCA-102 tumor cells were sensitive but also DCs were resistant to vincristine. Moreover, vincristine did not suppress the surface molecule expression on DCs such as CD11c, CD40, CD80 and CD86 molecules. These results indicated that vincristine could be used as the optimal chemotherapeutic agent for the combined chemotherapy and DC-base immunotherapy to develop an efficient antitumor strategy in the present study.

Similarly to the previous *in vivo* immunization studies using antigen-pulsed and unpulsed DCs (Fields et al., 1998; Candido et al., 2001; Tong et al., 2001), the therapeutic efficacy of the unpulsed, immature DCs delivered locally at the site of established MCA-102 tumor after pretreatment of vincristine was demonstrated in the present study. The growth of MCA-102 tumor cells was profoundly affected by the combination treatment with vincristine and DCs compared with that in the untreated group, the group treated with DCs alone, and the group treated with vincristine alone, especially after the second DC vaccination combined with vincristine pretreatment.

The results of the present study also demonstrated that expression of CD40, CD80 and CD86 costimulatory molecules was upregulated after the combined treatment with vincristine and immature DCs. Furthermore, the number of $CD4^+$ and $CD8^+$ T cells and the staining intensity of CD4 and CD8 surface molecules also increased after the combination treatment with vincristine and DCs.

These findings suggest that not only the immature DCs became activated and mature but also $CD4^+$ and $CD8^+$ T cell responses were induced *in situ* within the tumor after the combination treatment with vincristine and DCs. Tong et al. (2001) showed that the combination treatment with chemotherapy and intratumoral administration of DCs induced the generation of tumor-specific cytotoxic T cells that were able to protect animals from tumor challenge, and that the chemotherapy plus DC-treated animals were able to resist repeat challenge with the same tumor, suggesting that the mice had acquired long-term antitumor immunity.

Furthermore, similarly to the approach employed in this study for inducing tumor cell death using chemotherapy, Candido et al. (2001) induced tumor cell apoptosis using tumor necrosis factor α (TNF-α) and showed that this resulted in a greater DC-mediated antitumor effect. Thus, it is speculated that intratumoral DC administration combined with other tumor cell death-inducing agents without having significant toxic effects on normal cells and tissues *in vivo*, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), can be another novel, effective DC-based immunotherapeutic strategy (Walczak et al., 1999).

The strategy designed in this study has some obvious advantages including the following facts: (1) it does not require any surgical procedures to get tumor tissues for tumor antigens; (2) the doses of chemotherapeutic agents can be much more reduced than those for conventional chemotherapeutic methods; (3) this strategy may be a useful new approach to overcome the problem of drug resistance in conventional cancer chemotherapy; (4) this approach can be applicable to a wide variety of tumors; and (5) this

approach can be a more practical way in clinical use.

In conclusion, the efficacy of antitumor strategy was developed in the present study utilizing the combined effect of vincristine chemotherapy and DC-based immunotherapy against murine MCA-102 fibrosarcoma cells. Therefore, the evidence presented here shows that this novel antitumor immunotherapeutic strategy may be beneficial to vaccinate the cancer patients with their autologous, immature DCs in combination with conventional chemotherapies. Future studies are bound to improve this strategy for more effective and potent antitumor immunity.

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