

# Inhibitory effects of a dendritic cell vaccine loaded with radiation-induced apoptotic tumor cells on tumor cell antigens in mouse bladder cancer

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**ABSTRACT.** Herein, the preparation of a dendritic cell (DC) vaccine with radiation-induced apoptotic tumor cells and its immunological effects on bladder cancer in C57BL/6 mice was investigated. We used radiation to obtain a MB49 cell antigen that was sensitive to bone marrow-derived DCs to prepare a DC vaccine. An animal model of tumor-bearing mice was established with the MB49 mouse bladder cancer cell line. Animals were randomly allocated to an experimental group or control group. DC vaccine or phosphate-buffered saline was given 7 days before inoculation with tumor cells. Each group consisted of 2 subgroups in which tumor volume and the survival of tumor-bearing mice were recorded. Tumor volumes and average tumor masses of mice administered DC vaccine loaded with radiation-induced apoptotic cells were significantly lower than those in the control group (P < 0.01).

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Effects of DC vaccine with radiation-induced apoptotic cells

Survival in the experimental group was also longer than that in the control group, and 2 mice survived without tumor formation. In the DC vaccine group, 2 mice were alive without tumor growth after 30 days, and no tumor was observed at 30 days after subcutaneous inoculation of MB49 cells. The DC vaccine loaded with radiation-induced apoptotic tumor cells had an anti-tumor effect and was associated with increased survival in a bladder cancer model in mice.

**Key words**: Dendritic cells; Bladder tumor; Dendritic cell vaccine; MB49 cells

# **INTRODUCTION**

Dendritic cells (DCs) are powerful antigen presenting cells that can effectively stimulate a T cell response and induce generation of specific cytotoxic T lymphocytes. In China, bladder cancer is the most common malignant tumor of the urinary system with an incidence that ranks fifth among systemic tumors; transitional cell carcinoma accounts for 95% of all bladder cancers. The use of a DC vaccine to inoculate the host to induce or enhance host defense function, and thus improve the ability of the immune system to kill specific tumor cells, has become an intensely investigated research topic among immuno-therapies for advanced bladder cancer.

## **MATERIAL AND METHODS**

#### Cells and culture

MB49 cells were derived from bladder transitional cell carcinomas of female C57BL/6 mice induced by carcinogen (Department of Urology Research Institute, Huashan Hospital of Fudan University). The cell culture medium included 10% bovine serum, 1% penicillin/streptomycin, 0.1% sodium propionate, and 0.1% RPMI-1640. Cells were cultured at 37°C and 5% CO<sub>2</sub>.

## Animals

Animals were female C57BL/6 mice with an age ranging from 4-6 weeks (17-20 g). All mice were housed in a specific pathogen-free animal room.

## Preparation and characterization of bone marrow-derived DCs

Extraction of DCs was carried out using the method described by Inaba. In brief, the tibia and femur of 6-week-old healthy female SPF C57BL/6 mice were removed, and a 1-mL injector was used to flush out the marrow cavity with precooled phosphate-buffered saline (PBS). The filtrate was collected after filtration through a 200-hole single cell steel filter. After 5 min of centrifugation at a speed of 1200 rpm, the supernatant was discarded and 1 mL 40 g/L Tris-NH<sub>4</sub>Cl solution was used to resuspend cells. Cells were centrifuged again for 5 min at 1200 rpm to remove lysed red blood cells. The supernatant was discarded and cells were

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washed twice for 5 min with PBS. Cells were then resuspended in 10% RPMI-1640 containing fetal bovine serum (FBS) at a cell density of  $1.5 \times 10^6$  cells/mL and transferred to a 6-well culture plate with 2-mL wells. After 24 h of incubation, cells were washed twice with sterile PBS to remove non-adherent cells. Cells were then grown in the presence of recombinant murine granulocyte macrophage colony stimulating factor (10 µg/L) and recombinant mouse interleukin 4 (5 µg/L) in RPMI-1640 complete medium. Cells were separated by flow cytometry. Collected cells (density 5 x 10<sup>5</sup>/mL) were incubated in bovine serum albumin-PBS containing 2 µg monoclonal antibody (CD11c-APC, CD11b-PE, CDI-A-PE, CD80-PE, or CD46-PE) at 4°C for 30 min. After two washes with PBS, fluorescein isothiocyanate (FITC)-labeled anti-Goat rat IgG was added and the mixture was incubated for 30 min at 4°C. Cells were then washed with PBS twice and resuspended in PBS containing 1% paraformaldehyde. Flow cytometry (FACS Becton-Dickenson) was used for detection.

# Determination of irradiated tumor cells and apoptosis

In order to obtain apoptosis of tumor cells, cultured MB49 cells were collected and a single cell suspension was prepared at a density of 1 x 10<sup>7</sup> cells/mL before irradiation with 10,000 cGy (Faxitron CP-160, USA) (Hou et al., 2010). Irradiated cells were cultured in RPMI-1640 without FBS. Cells were stained daily with annexin V-FITC and propidium iodide and the proportion of apoptotic cells was determined by flow cytometry.

## Loading with tumor cell antigen

Irradiated MB49 cells were cultured in RPMI-1640 without calf serum for 3 days, mixed at a ratio of 1:1, and cultured with 1 x  $10^6$  immature DC for 24 h. The control group was immature DCs stimulated by lipopolysaccharide (LPS).

## Protective effect of DC vaccine

C57BL/6 female mice (N = 40) were randomly divided into 4 groups, which were treated with subcutaneous injection of PBS, immature DC cells, mature DC induced by LPS (LPS-DC), or irradiation. Co-cultured DC cells contained apoptotic MB49 cells (MB49-DC). Animals were innoculated with the DC vaccine once at 7 days followed by subcutaneous inoculation of MB49 tumor cells at 14 days. Each group was divided into 2 subgroups for the measurement of tumor volume and survival of tumor-bearing mice.

## **Statistical analysis**

All data were analyzed with the SPSS 10.0 software. Data are reported as means  $\pm$  SD. A *t*-test was used to compare groups. A P  $\leq$  0.05 was considered to be statistically significant.

## RESULTS

#### A large number of DCs can be obtained by culture *in vitro*

Stem cells derived from bone marrow can be induced to differentiate into DCs with

immunological activity. Study of the immune response induced by loaded tumor antigen requires a sufficient amount of DCs at the same level of maturity. To obtain these, we first amplified bone marrow derived stem cells from mice *in vitro* by adding IL-4 and GM-CSF to culture medium (Figure 1). After 5 days, high levels of expression of CD11c were detected by surface antibodies, but low levels of expression of CD80, CD86, and I-A were seen, which suggested that these were immature DC cells. Detection of CD80, CD86, and I-A after co-culture with LPS for 7 days suggested that the DCs were mature (Figure 2).



Figure 1. Cells cultured for 5 days in mouse bone marrow-derived DCs.



**Figure 2**. Flow cytometry. Cells were cultured for 5 days. Surface antibody showed high expression of CD11c, but low expression of CD80, CD86, and IA, suggesting that DCs were immature. LPS was added and cells were co-cultured for 7 days and CD80, CD86, and I-A were detected, suggesting maturation of DCs. (BM-DC: bone marrow derived DCs; LPS-DC: bone marrow-derived cells after co-culture with LPS).

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# Apoptotic tumor cells are obtained after irradiation

In order to obtain the optimal number of apoptotic cells, we measured the tumor cells that were irradiated and cultured in medium without serum by flow cytometry on a daily basis. We found that apoptosis of tumor cells reached optimal numbers after 3 days (Figure 3).



Figure 3. After X-ray irradiation, tumor cell apoptosis reached an optimum number after 3 days.

# Mature DCs are obtained by co-culture of apoptotic tumor cells and immature DCs

Figure 4 shows the results after co-culture immature DCs and DCs after irradiation for 5 days and culture in serum-free medium for 1 day. Flow cytometry showed that DCs had matured.



Figure 4. After co-culture for 1 day with immature DCs and apoptotic tumor cells, flow cytometry tests showed that immature DCs mature.

# Protective effect of DC vaccine

In order to determine whether the DCs loaded with apoptotic tumor cells could function as a vaccine, we used a subcutaneous tumor model (Hou et al., 2010). As shown in Figure 5, when PBS is injected, growth of tumors in mice is very fast and leads to rapid death. Tumor growth in mice previously injected with the immature DC and LPS-DC was slower, but caused

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death in all mice. However, tumor growth was very slow in mice receiving injection of MB49-DC. In addition, two mice survived without tumor (Figure 6).



Figure 5. Effects of DC vaccine on tumor burden in mice.



Figure 6. MB49-DC vaccination significantly slowed tumor growth in mice. Of note, tumors did not grow in 2 mice.

## DISCUSSION

DCs are powerful antigen presenting cells. DCs can capture and process antigens, and then migrate to lymphoid organs to activate T lymphocytes, especially naive T cells and resting T cells. The cellular immunity mediated by T lymphocytes, and in particular cytotoxic T lymphocytes, may play an important role in anti-tumor and anti-infective responses (Morel and Turner, 2010). Biological treatment with DCs loaded with tumor antigens is a promising vaccine (Koido et al., 2010). Numerous *in vivo* and *in vitro* studies have confirmed that such treatment can generate strong anti-tumor immunity and inhibit the growth and metastasis of tumors after application of different forms of tumor antigen *in vitro* to sensitized DCs.

Several *in vivo* and clinical trials have been conducted on DC vaccine (Steinman and Pope, 2002; von Euw et al., 2008). In exploring a tumor vaccine, researchers have used a variety of strategies to develop a DC vaccine that can stimulate a tumor-specific immune response. These have mainly included DCs loaded with tumor antigens, tumor antigen sensitized DCs, and tumor antigen gene transfection DCs (Yanofsky et al., 2013). A DC vaccine with a tumor cell lysate can induce a cytotoxic T lymphocyte response, lyse tumor cells, and cause secretion of high levels of interferon- $\gamma$  (Li et al., 2008). However, the antigenicity of cell antigens is poor as it contains many non-tumor associated antigens. A whole cell antigen has multiple epitopes and induced cytotoxic T lymphocyte clones for different antigenic determinants, which is more amenable to immune attack of tumor cells. Fry et al. (2009) compared various methods and showed that after co-culture of apoptotic tumor cells induced by irradiation with immature

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DCs, the latter cells can devour apoptotic bodies and process tumor antigens, which can greatly improve the antitumor activity of DCs. According to Hoffmann et al. (2000), it may produce more autologous CD8+ cytotoxic T lymphocytes using apoptotic cells compared with lysates.

Our study suggests that a large number of high quality DCs can be obtained by culture *in vitro*, thus providing the possibility for DC treatment. However, immature DC cells do not have a vaccine function and thus cannot be used as treatment. Moreover, mature DC cells induced by LPS also have no vaccine effect because no tumor antigen has been presented.

Numerous apoptotic cells can be detected in tumor cells after irradiation. On one hand, ionizing radiation can penetrate the inside of tumor cells and destroy DNA, inhibiting or killing tumor cells. On the other hand, radiation can improve the immunogenicity of tumor cells by inducing them to release HMGB-1, HSP70, and other endogenous "danger signals", and can also upregulate the expression of MHC-I, ICAM-1, VCAM-1, and other adhesion molecules, thereby promoting chemotaxis of immune cells and activating the innate immune and adaptive immune responses of the host. Apoptosis of tumor cells can induce a specific immune response to antigens. In addition, a mature reaction may appear after DCs devour apoptotic cells, which upregulate chemokines, cytokines, and co-stimulatory molecules. Albert et al. (2001) demonstrated that DCs incubated with apoptotic cells can generate tumor-specific cytotoxic T lymphocytes, while necrotic cells cannot.

Our results suggest that a large number of apoptotic cells can be detected after irradiation of tumor cells, which can produce high purity mature DCs when incubated with immature DC cells. The mature DC cells induced by apoptosis of tumor cells have a vaccine effect. Our experiments show that this type of DC has an antitumor effect on bladder cancer in C57BL/6 mice.

## CONCLUSIONS

DC vaccine loaded with radiation-induced apoptotic tumor cells has an antitumor effect and prolongs survival in a mouse model of bladder cancer.

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