Enhancement of dendritic cells with melanoma-associated antigen 3 for inducing cytotoxicity by cytotoxic T lymphocytes on bladder cancer BIU-87 cells

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ABSTRACT. To determine the cytotoxic effect of lymphocytes activated by melanoma-associated antigen 3 (MAGE-3)-sensitized dendritic cells (DCs) on BIU-87 tumor cells, and to evaluate the

possibility of MAGE-3-peptide-pulsed DCs as a vaccine in bladder cancer immunotherapy, the proliferation of T cells and the activity of cytotoxic T lymphocytes (CTLs) were examined by the MTT method. CTLs were induced by MAGE-3-sensitized DCs, or by ovalbumin (OVA) peptide and non-sensitized DCs as controls, respectively. The results indicated that MAGE-3-sensitized DCs have the ability to promote the proliferation of T cells as well as the cytotoxic activity of CTLs on bladder cancer cells in comparison with OVA peptide and non-sensitized DCs. In other words, DCs sensitized by the MAGE-3 antigen peptide could obviously upregulate the proliferation of T cells, which resulted in the growth inhibition of bladder cancer BIU-87 cells. In addition, MAGE-3-sensitized DCs played an important role in inhibiting the growth of human BIU-87 tumor xenografts in nude mice.

Key words: Bladder neoplasms; Dendritic cells; Peptide; MAGE-3; Vaccine

INTRODUCTION

A number of melanoma-associated antigen (MAGE) genes have been demonstrated in different types of tumors, ever since Van der Bruggen et al. (1991) found the first MAGE and its peptide MZ2-E in 1991. Most of these genes are mapped to chromosome X and share certain homologous regions. Members of the MAGE family include subgroups A, B, C, D, E, F, etc. Specifically, subgroups A, B, and C share a certain degree of homology, as determined from their expression in different types of malignant tumors (Yakirevich et al., 2003; Xiao et al., 2005; Haier et al., 2006; Peikert et al., 2006; Jacobs et al., 2007; Picard et al., 2007). However, these genes have not been detected in normal adult tissues except in the testis and the placenta. Although the signaling pathway and physiological function of proteins encoded by the MAGE genes have not been elucidated clearly, they could be recognized by specific T cells as tumor antigens in malignant tumors. The aforementioned studies all showed that although the MAGE family is likely to become novel markers for tumor detection, its antigen peptide may be the most promising candidate for tumor-specific immunotherapy of cancers.

Dendritic cells (DCs) act as antigen-presenting cells for activating naïve T cells and initiating a primary immune response. Recently, increasing evidence has indicated that cellular immunity is induced by DCs in response to a malignant tumor. Hence, efforts need to be directed towards adapting cellular immunotherapies based on antigen-loaded DCs (Rossowska et al., 2007; Michiels et al., 2008). We conclude that antigen-specific cytotoxic T lymphocytes (CTLs) induced by MAGEs can be applied for the treatment and prevention of hepatocellular carcinoma.

MATERIAL AND METHODS

Main reagents

RPMI 1640 medium (Lot 1342878; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), L -glutamine (Lot 2E1026; JRH Biosciences, Lenexa, KS, USA), Trizol reagent (Lot

40224; Canada Bio Basic Inc., Markham, ON, Canada), RNA enzyme inhibitors (Shanghai Public Health, Shanghai, China), M-MLV reverse transcriptase (Lot 00014891; Fermentas, Waltham, MA, USA), random primer oligo(dt)18 (Shanghai Biological Engineering Co., Ltd., Shanghai, China), TaqE (Lot E4221; Tiangen, Beijing, China), dNTP (Shanghai Biological Engineering Co., Ltd., Shanghai, China), interleukin 4 (IL-4) (Peking University Health Science Department, Beijing, China), granulocyte macrophage colony-stimulating factor (GM-CSF) (Lot CYT221; Prospec Chemicals, Fort Saskatchewan, AB, Canada), Hoechst 33258 solution (Lot 94403; Sigma-Aldrich Co., LLC., Saint Louis, MO, USA) and tumor necrosis factor-alpha (TNF-α) (Lot 01616; Shanghai Celstar Bio-Pharmaceutical Co., Ltd., Shanghai, China) were all procured from the respective suppliers. MAGE-3 (a 9-amino-acid peptide, LLIIVLAII) and ovalbumin (OVA; a 9-amino-acid peptide, YSIINFEKL) were synthesized by Beijing Keya Biological Technology Co., Ltd. (Beijing, China) at 95% purity.

Cell culture and RPMI 1640 medium preparation

One bag of RPMI 1640 powder, 2.0 g of $NaHCO_3$, and 2.4 g of Hepes were dissolved in 1 L of double-distilled complement in bottles. The medium was filtered to remove bacteria. To prepare calf-serum-containing medium, 50 mL of inactivated calf serum, 5 mL of double antibiotics (penicillin and streptomycin at final concentrations of 1 x 10^4 U/mL), and 5 mL of L-glutamine (30 mg/mL) were added to 440 mL of RPMI 1640 medium, and the pH was adjusted to 7.2 with 10% $NaHCO_3$. Recovery of the human bladder cancer cell line BIU-87 was done in RPMI 1640 complete medium containing 10% calf serum, 1% L-glutamine, and 1% double antibiotics, with incubation at 37°C in a 5% CO_2 incubator.

MAGE-3 mRNA expression in BIU-87 cells

Total RNA was isolated from BIU-87 cells using the acid guanidinium thiocyanate-phenol chloroform extraction procedure. cDNA synthesis from 2.5 mg of total RNA was performed by extension with oligo(dT)18 in a 20 μL reaction volume. MAGE cDNA samples were detected by polymerase chain reaction (PCR) amplification by conducting 35 cycles (45 s at 94°C and 60 s at 72°C) using oligonucleotide primers. To determine the expression of MAGE-3 in urinary bladder carcinoma cells, the following primer pairs were used: MAGE-3, 5'-CAG CCA ATA ATC ACA ACC-3' and 5'-GAG ACA TAA TCC AGC ACC-3' (515 bp); and β-actin, 5'-CCA CGA AAC TAC CTT CAA CTC C-3' and 5'-CAT ACT CCT GCT TGC TGA TCC-3' (266 bp). The following primer pairs were used to determine the expression of MAGE-3 in the BIU-87 cell line: MAGE-3, 5'-TGG AGG ACC AGA GGC CCC C-3' and 5'-GGA CGA TTA TCA GGA GGC CTG C-3' (500 bp); and β-actin, 5'-GGT ATG GGT CAG AAG GAC TCC-3' and 5'-TGA TCT TCA TGG TGC TAG GAG CC-3' (318 bp). The PCR products were size-fractionated on a 1% agarose gel.

Generation of dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of a healthy HLA-A2 donor, separated by Ficoll-Hypaque density gradient centrifugation (Shanghai Hang Shun Chemical Co., Ltd., Shanghai, China), and then washed three times with phosphate-buffered saline (PBS). Monocyte-derived DCs were generated under serum-

free conditions from the adherent fraction of PBMCs cultured in six-well plates at a density of 5 x 10^6 cells/mL for 2 h at 37°C and 5% CO_2. The non-adherent cells were removed. The adherent cells were resuspended in RPMI 1640 medium supplemented with 1000 U/mL GM-CSF and 1000 U/mL IL-4 and incubated in a humidified incubator at 37°C and 5% CO_2 for 5 days. On the 6th day, maturation of DCs was induced by addition of 1000 U/mL TNF-α for 2 days, DC phenotype was characterised by staining 5 x 10^6 cells with fluorochrome-labeled antibodies against the Lin1 panel (CD1a, CD80, CD83, and CD86) and analyzing the cells by fluorescence-activated cell sorting (FACS) Calibur flow cytometry.

MAGE-3-sensitized DCs

DCs (3 x 10^6 cells/mL) were incubated with MAGE-3 peptide or OVA peptide for 24 h at 37°C. RPMI 1640 medium without peptide served as the negative control group.

Purification and identification of T lymphocytes

PBMCs were isolated from venous blood of a healthy HLA-A2 donor by Ficoll-Hypaque density gradient centrifugation, and T cells were then collected by purification through a nylon wool column. The T cells were identified by flow cytometry (T cells >80%).

T lymphocyte proliferation test

After adjusting the T lymphocyte concentration to 2 x 10^5 cells/mL, 100 μL of the cells pulsed with DCs (T:DC = 25:1) was added to 100 μL of MAGE-3 or OVA, respectively, in 100 μL of RPMI 1640 medium in 96-well plates. The plates were incubated for 72 h at 37°C and 5% CO_2. Before the end of 4 h, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and incubation was continued for a further 4 h. The supernatant was removed after centrifuging for 10 min at 2000 rpm. Dimethyl sulfoxide (150 mL) was added to dissolve the cells completely. Activated T-cell proliferation was detected by absorbance measurement. The control group contained only T lymphocytes and RPMI 1640 complete medium.

Antitumor activity of CTLs

After concentration adjustment to 2 x 10^5 cells/mL, 100 μL of T lymphocytes pulsed with DCs (T:DC = 25:1) was added to 100 μL of MAGE-3 or OVA, respectively, in 100 μL of RPMI 1640 medium in 96-well plates, and incubated for 72 h at 37°C and 5% CO_2. Then, 100 μL of BIU-87 (at 2.5 x 10^4 and 1.25 x 10^4 cells/mL) was added and incubation continued for 48 h. The CTL antitumor activity was determined by the MTT assay. The control group consisted of T lymphocytes and RPMI 1640 complete medium.

Detection of apoptosis induction by flow cytometry

BIU-87 cells of the different test groups were cleaned twice using PBS buffer and fixed for 24 h in 70% cold ethanol at 4°C. The ethanol was removed and the tumor cells were cleaned once using PBS buffer. Then, 200 μg/mL of RNA enzyme was added, and the cell-

stage distribution of the different DNAs was determined by flow cytometry. The data were processed with the ModifitL TL 1.00 (MAC) analysis system. Apoptotic cells were lower in number than cells in the G1 phase. The apoptosis rate was the proportion of the apoptotic cells to the total number of cells.

Detection of apoptosis-related proteins by FACS

BIU-87 cells (1 x 10^6 cells/mL) treated with MAGE-3 were cleaned twice using PBS buffer, and then fluorescein-isothiocyanate-labeled Bcl-2 antibody or FAS antibody was added. The reaction was conducted for 30 min in the dark. Thereafter, the cells were cleaned once using PBS buffer and apoptosis-related proteins were detected by flow cytometry. Untreated cells were used as the negative control.

Antitumor effects of MAGE-3-sensitized DCs on human BIU-87 tumor xenografts in nude mice

Twelve tumor-bearing nude mice were divided into three groups (N = 3, N = 4) to be tail-vein-injected with l x 10^6 DCs and/or T lymphocytes. Group A was the model control group injected with physiological brine only, group B was injected with T lymphocytes only, and group C was injected with CTLs and MAGE-3-sensitized DCs. The antitumor effect was indicated by changes in the tumor weight and tumor volume on the 7th day.

Hoechst assay

The cells by centrifugation were stuck on to glass slides with 4% paraformaldehyde for 10 min, washed in two changes of distilled water and ready to dry at room temperature. Add Hoechst 33258 (5 mg/L) for 5 min, then washed in two changes of distilled water. Next, dry at room temperature. Observe and take pictures randomly under the fluorescence microscope.

Tunel assay

The cells were stuck on to glass slides with 4% paraformaldehyde for 30-60 min. Then the slides were washed in PBS and distilled water 2 times respectively, treated with by both 3% H_2O_2 and distilled water 2 min for 2 times respectively. Next, the slides were partially digested with proteinase-K (20 μg/mL) for 15 min, washed in two changes of PBS, incubated with equilibration buffer for 5 min, followed by incorporation of 1 μL dUTP-digoxigenin in the presence of 1 μL TdT per slice for 60 min in a humidified chamber at 37°C. Add 50 μL blocking reagent per slice at room temperature for 30 min. Use the antibody diluent that the antibody accounts for 1 percentage to dilute dUTP-digoxigenin antibody and put it on the slice with blending. Then the slides were placed in a humidified chamber with 37°C for 30 min, washed by PBS 2 min for 3 times. Use the antibody diluent that the antibody accounts for 1 percentage to dilute SABC and put it on the slice with blending. Then the slides were placed in a humidified chamber with 37°C for 30 min, washed by PBS 5 min for 4 times. Blend one drip of A,B,C of DAB kit respectively with the 1 mL distilled water. Then add the blending liquid into the specimen 10-30 min and washed with distilled water. The slides were mild redyed with haematein, washed with PBS and distilled water. After dehydration, transparency and

sealing, the slides can be observed with the microscope. Detect 10 cells at high magnification randomly and calculate the percentage of positive cells. Repeat 3 times for the above steps and get the average for the apoptosis index (AI).

Statistical analysis

All experimental data were analyzed using the SPSS 10.0 statistical package.

RESULTS

Expression of MAGE-3 in urinary bladder tumor and BIU-87 cells

The expression of MAGE-3 in urinary bladder carcinoma was detected by immunohistochemical staining assay and indicated with different staining grading (Figure 1A and B indicated as staining grading +++ and staining grading +). Total RNA was isolated from urinary bladder tumor or BIU-87 cells by the acid guanidinium thiocyanate-phenol chloroform extraction method. In addition, the expression of MAGE-3 was measured by RT-PCR assay, using different primers. The results indicated that the expression of MAGE-3 was upregulated in urinary bladder tumor cells and in BIU-87 cells, compared with normal control cells (Figure 1C for urinary bladder tumor cells, and Figure 1D for BIU-87 cells).

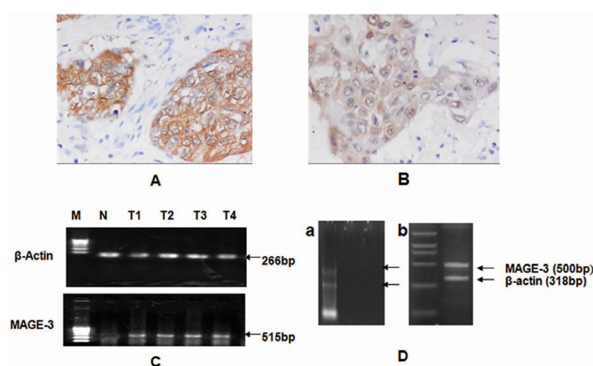

Figure 1. A. Expression of MAGE-3 in urinary bladder carcinoma (immunohistochemical staining grading +++); 200X magnification. **B.** Expression of MAGE-3 in urinary bladder carcinoma (immunohistochemical staining grading +); 200X magnification. **C.** Expression of the MAGE-3 gene in renal tumor, urinary bladder tumor, and tumor-surrounding tissues (lane M: DNA ladder marker; lane N: N-urinary bladder carcinoma surrounding tissues; T1: T1-renal pelvis carcinoma; T2: T2-urinary bladder carcinoma; T3: T3-urinary bladder carcinoma; T4: T4-renal carcinoma). **D.** Expression of MAGE-3 in N-urinary bladder carcinoma BIU-87 cells (**a.** 28S:18S; **b.** MAGE-3).

Purity and maturity of MAGE-3-sensitized DCs

PBMCs were isolated from venous blood of a healthy HLA-A2 donor and cultured under serum-free condition for induction of DCs. The DCs were then sensitized with MAGE-3 as described in the Materials and Methods section. Characterization of the DC phenotype (CD83, CD86, and CD11c) was conducted by flow cytometry. The results indicated that the DC purity was about 91.09% and maturity was 11.92%. This high yield of matured DCs could be used to study their functions in inducing and activating CTLs (Figure 2).

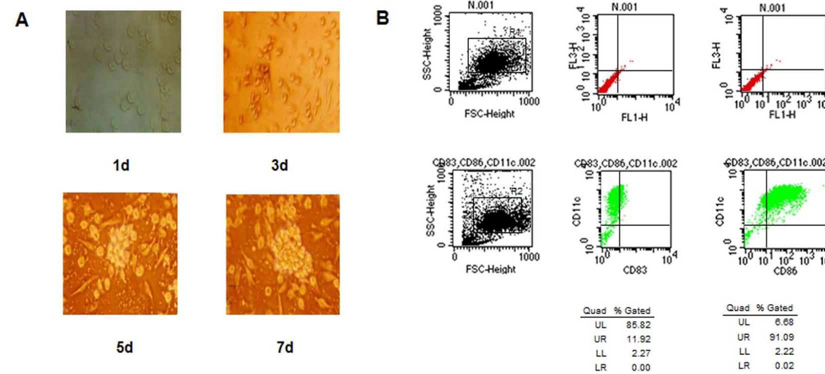

Figure 2. Determination of the purity of dendritic cells (DCs) by FACS analysis, with grade of 91.09% purity and 11.92% maturity. **A.** DC morphology. **B.** FACS quantitation of DC markers.

DC-induced T lymphocytic proliferation and CTL cytotoxicity

T lymphocytes were exposed to MAGE-3-sensitized DCs and their proliferation was detected by MTT assay. Activation of the T lymphocytes was evident (Figure 3). The antitumor activity of CTLs was also detected by the MTT method, revealing an increase in the T-lymphocyte proliferation rate. It was concluded that the MAGE-3 antigen peptide had a significant effect on activating CTLs, compared with the effects of the OVA antigen peptide and no-peptide control (P > 0.05).

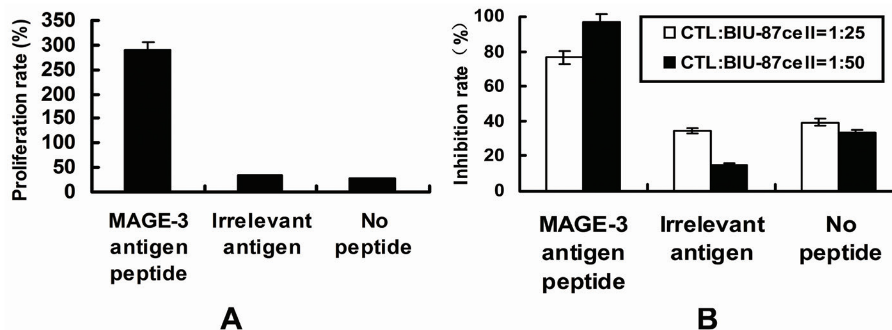

Figure 3. A. Effect of MAGE-3 on T lymphocyte proliferation. **B.** Activity of cytotoxic T lymphocytes (CTLs) induced by MAGE-3-sensitized dendritic cells on BIU-87 cells.

Apoptosis induction of BIU-87 cells by CTLs

Apoptosis of the tumor cells was significantly induced by the MAGE-3-sensitized DC antigen, showing an apoptosis rate of 24.94% (Figure 4). The control group treated with OVA-sensitized DCs and the group with untreated T lymphocytes alone could not induce obvious apoptosis, showing apoptosis rates of only 1.82 and 1.42%, respectively. The results verified that MAGE-3 antigen peptide vaccines could induce tumor cell apoptosis as a mechanism of killing tumor cells.

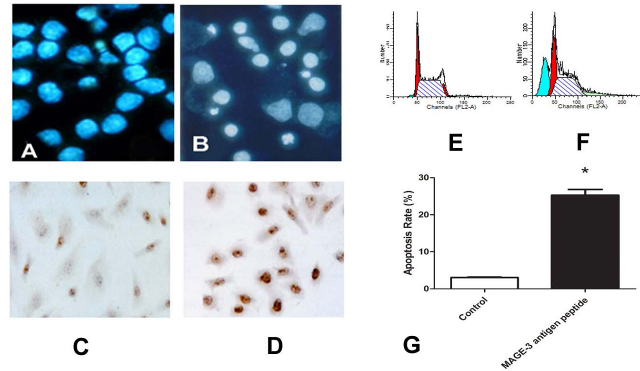

Figure 4. Apoptosis of BIU-87 cells. **A.** Hoechst assay for the control group. **B.** Hoechst assay for the MAGE-3 peptide group. **C.** TUNEL assay for the control group. **D.** TUNEL assay for the MAGE-3 peptide group. **E.** FACS assay for the control group. **F.** FACS assay for the MAGE-3 peptide group. **G.** Average rate of apoptosis (*P < 0.05).

Expression of apoptosis-related genes

Apoptosis-related proteins were detected by FACS assay. Fas expression in the MAGE-3 (Treated) and OVA (Control) peptide groups was 15.7 and 9.36%, respectively, whereas that of Bcl-2 was 5.3 and 1.96%, respectively (Figure 5). Through statistical analysis, Fas expression was determined to be obviously upregulated, and Bcl-2 expression downregulated, in MAGE-3-sensitized DC-activated CTLs relative to the model control group.

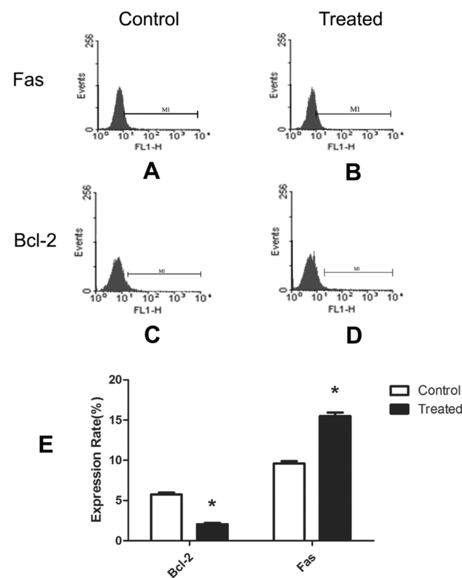

Figure 5. Effect of cytotoxic T lymphocytes sensitized by MAGE-3 antigen peptide on Fas and Bcl-2 expression in tumor cells. **A.** Fas expression in the irrelevant antigen peptide (Control) group. **B.** Fas expression in the MAGE-3 antigen peptide (Treated) group. **C.** Bcl-2 expression in the irrelevant antigen peptide (Control) group. **D.** Bcl-2 expression in the MAGE-3 antigen peptide (Treated) group. **E.** Graphical comparison of the expression rates of both proteins (*P < 0.05).

Role of MAGE-3-sensitized DCs in inhibiting growth of human BIU-87 tumor xenografts in nude mice

Twelve nude BIU-87 xenograft mice were randomly divided into the BIU-87 xenograft model group, T lymphocytes-only group, and MAGE-3-sensitized DC-activated CTL group. The tumor volume and weight were measured at 7 days post treatment. The tumor size of each group demonstrated obvious changes (Figure 6). More specifically, both the tumor volume and weight of the MAGE-3-sensitized group were less than the T lymphocytes-only group and model control group (P < 0.05). The result indicated that MAGE-3-sensitized DC-activated CTLs were able to inhibit the growth of the BIU-87 xenograft tumor significantly. In contrast, the T lymphocytes-only group had no significant therapeutic effect on BIU-87 xenograft tumors *in vivo*, which was consistent with the *in vitro* experiments.

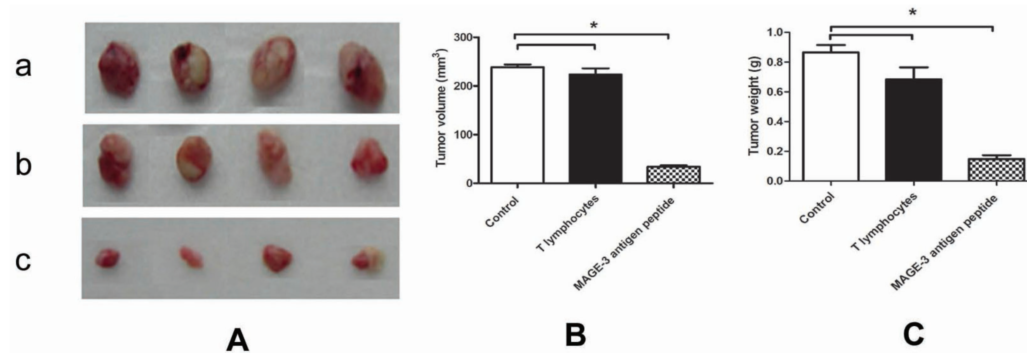

Figure 6. Effect of cytotoxic T lymphocytes sensitized by MAGE-3 antigen peptide *in vivo*. **A.** Comparison of tumor sizes (a: control; b: T lymphocytes; c: MAGE-3 antigen peptide). **B.** Comparison of average tumor volumns after 7 days of treatment. **C.** Comparison of average tumor weights after 7 days of treatment. *P < 0.05.

DISCUSSION

MAGE genes can be expressed in various cancers. Its expression renders MAGE a possible valuable target in tumor-specific immunotherapy. Patard et al. (1995) reported that MAGE-1- and MAGE-3-positive expression was 21 and 35%, respectively, in transitional-cell carcinomas of the urinary bladder. Moreover, the expression percentage was upregulated in advanced stages of the cancer. In our preliminary experiments, we concluded that both bladder and upper tract urothelial cancers show high levels of MAGE-1 and MAGE-3 expression (Ma et al., 2004).

As a result, with respect to treating bladder cancer, MAGE could be a functional target for activating immunotherapy. The objective of this study was to develop effective immunotherapies for bladder cancers. We hypothesized that combining autologous DCs with the HLA-A2-specific MAGE epitope peptide should be an effective way to guide autologous CTLs against MAGE expression in these cancers. Both innate and adaptive immunities comprise a coordinated mechanism for antitumor immunity, which is primarily mediated by natural killer (NK) cells, natural killer T (NKT) cells, and cytotoxic T cells. DCs, which can regulate, coordinate, and induce immune response, play an important role within this context (Banchereau et al., 2000). As antigen-presenting cells in tissues or peripheral blood, DCs are involved in the uptake and processing of antigens. They usually migrate to lymph nodes and

present antigens to T lymphocytes, which then induce the activation of CD4+ T cells or CD8+ T cells. Moreover, the humoral immune system has the function and ability to activate naïve and memory B cells via DC induction (Jego et al., 2003). In addition, NK and NKT cells may be activated by DCs (Kadowaki et al., 2001). Because they modulate the entire immune repertoire, DCs have the ability to execute outstanding function against cancers. Therapeutic cancer vaccines not only activate the immune system to treat an existing tumor, but also have the ability to prevent its recurrence.

In the present study, it was demonstrated that DCs pulsed with the HLA-A2-binding MAGE-3 epitope peptide could induce a CTL response, and these CTLs could recognize MAGE-3-positive tumor cells in an MHC class I-restricted manner; for example, CTLs from a healthy donor with HLA-A2 type had the ability to kill BIU-87 cells. The results of this study show that there is a significant kill-effect on the bladder cancer cell line BIU-87 by CTLs that were activated by DCs pulsed with the MAGE-3 antigen peptide. In contrast, both CTLs activated by DCs pulsed with the irrelevant OVA antigen and T lymphocytes activated by non-pulsed DCs did not demonstrate the killing action on BIU-87 cells and target cells, respectively. To further explore the mechanism of destruction of tumor cells, we determined changes in the expression of apoptosis-related proteins in BIU-87 cells by flow cytometry. To further explore the mechanism of apoptosis of tumor cells, we determined the changes of apoptosis-related proteins in BIU-87 cells by flow cytometry, the results indicated that the expression of Fas was up-regulated and the expression of Bcl-2 was down-regulated, and the ratio of Fas and Bcl-2 was up-regulated obviously, which contributed to the induction of apoptosis of BIU-87 cells.

Furthermore, the role of MAGE-3-sensitized DCs was investigated using the BIU-87 tumor xenograft nude mice model. Taken together, the results indicated that MAGE-3-sensitized DC-activated CTLs play a role in inhibiting the growth of BIU-87 tumor cells, both *in vitro* and *in vivo*.

In conclusion, T lymphocytes activated by DCs pulsed with the MAGE-3 antigen peptide could induce killing action on bladder cancer cell lines; thus, the MAGE-3 gene could be a target for tumor immunotherapy. Combining the MAGE-3 antigen peptide with DCs could be an effective tumor vaccine for bladder cancer immunotherapy.

Conflicts of interest

The authors declare no conflict of interest.

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