Immunotherapy of Bladder Cancer Using Autologous Dendritic Cells Pulsed with Human Lymphocyte Antigen-A24-specific MAGE-3 Peptide

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ABSTRACT

Recent investigations have demonstrated the efficacy of autologous dendritic cells (DCs) pulsed with tumor antigens to generate tumor-specific CTLs against cancer cells. Melanoma antigens (MAGE) are a family of tumor-specific antigens shown to be expressed in various tumors, including bladder cancers and melanoma, but not in normal tissues except for the testis. Because invasive bladder cancers are frequently reported to express MAGE, we explored the possibility of establishing a new immunotherapeutic modality against advanced bladder cancer using autologous DCs pulsed with one of the MAGE-3 epitope peptides (IMPKAGLLI), which is synthesized to bind specifically to HLA-A24. A MAGE-3-expressing bladder cancer cell line, FY, was newly established from a lymph node metastasis of bladder cancer in a HLA-A24+ patient. The FY cell-specific CTL response was significantly higher when CTL was induced by autologous DCs pulsed with IMPKAGLLI than by FY cells alone or by nonpulsed DCs in vitro. A total of four HLA-A24+ patients with advanced MAGE-3+ bladder cancers were treated with s.c. injections of autologous DCs pulsed with IMPKAGLLI every 2 weeks for a minimum of 6 and a maximum of 18 times. Three of four patients showed significant reductions in the size of lymph node metastases and/or liver metastasis. No significant untoward side effects were noted in these patients. This study indicated that, at sometime in the future, tumor-specific DC-based cancer immunotherapy may be useful as an additional treatment modality against advanced bladder cancer.

INTRODUCTION

Several tumor antigens recognized by CTLs have been identified in multiple types of solid tumors (1). Many of these antigens are derived from tissue-specific differentiation antigens (2, 3), from oncogenes (4, 5), or from a set of antigens expressed preferentially in tumors (6–9). Because of recent progress in understanding tumor-specific antigens that can potentially stimulate CTL and Th2-cell responses, efforts to develop peptide- and cell-based tumor vaccines are increasing (10, 11). Melanoma antigens such as the MAGE family are now well known as tumor-rejection antigens recognized by CTLs in a HLA-restricted manner (12–14). MAGE is reported to be expressed in a broad range of cancers including melanoma (6), esophagus (15), breast (16), lung (17), and bladder (18) but not in normal tissues except for the testis, which does not express HLA class I molecules (19, 20). Therefore, these antigens are considered to be attractive targets for anticancer immunotherapy. DCs are the most potent professional antigen-presenting cells for inducing antitumor immunity both in vitro and in vivo. Several clinical studies already have demonstrated the potential efficacy of active immunotherapy using DCs loaded with various tumor-specific antigens in vitro (21–24).

Bladder cancers constitute a broad spectrum of malignancies at clinical presentation. Various chemotherapies with multiple anticancer reagents have only limited efficacy against highly advanced disease and have considerable systemic side effects (25). On the other hand, superficial bladder cancer, especially carcinoma in situ disease, is well known to respond to immunotherapy such as intravesical treatment by Bacillus Calmette-Guérin, and thus tumor-specific immunotherapy has been suggested as a potentially useful strategy against bladder cancer (26). In the present study, we were able to establish a MAGE-3+ bladder cancer cell line from a HLA-A24+ patient with advanced bladder cancer. Recently, Tanaka et al. (14) reported the successful induction of antitumor CTLs with a MAGE-3-encoded synthetic peptide presented by HLA-A24. In this previous report, five peptides of nine amino acids were found to contain the binding motif for HLA-A24 in the known sequence of MAGE-3. These peptides were synthesized and tested for their binding ability to purified HLA-A24 molecules. Of the five peptides studied, one high MHC binder (IMPKAGLLI) was found to be capable of eliciting CTLs. Using this particular model, we explored the possibility of inducing a

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2 The abbreviations used are: Th, T-helper; DC, dendritic cell; MAGE, melanoma antigens, RT-PCR, reverse transcriptase-PCR; HLA, human lymphocytic antigen; PBMC, peripheral blood mononuclear cell; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; mAb, monoclonal antibody.
significant MAGE-specific CTL response by the in vitro stimulation of PBMCs with autologous DCs pulsed with the MAGE-3 synthetic epitope peptide, IMPKAGLLI, which binds to HLA-A24 molecules with a very high affinity. On the basis of this achievement, a pilot clinical trial of DC vaccination targeting MAGE-3 has been conducted in selected HLA-A24+ patients with advanced bladder cancer proven to be MAGE-3+.

MATERIALS AND METHODS

Cell Lines. Five established human bladder cancer cell lines, KU-1, KU-7 (27), KU-19-19 (28), T24 (29), and a newly established FY cell line were used. The cell line was established from the lymph node metastasis of a bladder cancer in a 76-year-old HLA-A24+ Japanese female patient. In March, 1996, the patient underwent a radical cystectomy for muscle invasive bladder cancer. The histological evaluation revealed a TCC = SCC > AC, grade 3, PT3, nonpapillary invasive tumor. The HLA typing of this patient was A24 (9), B7, B52 (5), and Cw7. Metastases to the right inguinal and para-aortic lymph nodes were apparent in August, 1996. A primary culture was established from the lymph node metastasis of a bladder cancer. K562 cell line was kindly supplied from the Japanese Cancer Research Bank (Tokyo, Japan). The cell lines were well maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics.

Clinical Tumor Samples. Specimens of the 28 bladder cancers and 8 upper tract urothelial cancers as well as normal bladder mucosa samples were obtained from the patients at the time of surgery performed at Keio University Hospital. A routine histopathological evaluation was conducted, and a portion of each sample was immediately frozen in liquid nitrogen and stored at −80°C for later RNA extraction.

Analysis of mRNA Expression. Total RNA was isolated from each of the samples using the acid guanidinium thiocyanate-phenol chloroform extraction procedure (30). As described by De Smet et al. (31), cDNA synthesis from 2.5 μg of total RNA was accomplished by extension with oligo(dT)15 in a 20-μl reaction volume. Each of the MAGE cDNA samples was detected by PCR amplification by 33 cycles (30 s at 94°C and 40 s at 72°C) using oligonucleotide primers specific for the different exons of each MAGE gene. The sequences were as follows: (a) 5′-CCGCCGAAGAACCTGACCG-3′ (CHO-14) and 5′-GCTGAAACCCTCAGGTGTGC-3′ (CHO-12) for the MAGE-1 gene (16); (b) 5′-AATGAGCCGCCAG-GCACTG-3′ (CDS-9) and 5′-GAGACGGAGAACCGCTG-3′ (CDS-7) for the MAGE-2 gene (31); and (c) 5′-TGAGGACCGAGGCCC-3′ (AB-1197) and 5′-GGACGATT-TATCAAGGGCTGC-3′ (BLE-5) for the MAGE-3 gene (17). To ensure that the RNA had not degraded, a PCR assay for the glyceraldehyde-3-phosphate dehydrogenase gene was also carried out; the sense primer was 5′-GTAACAGGATTGGTGCTATTT-3′ and the antisense primer was 5′-AGTCTTCTTGGTGGCAGTGAT-3′ (32). The PCR product was size-fractionated on 1% agarose gel.

Statistical evaluations regarding the relationship between the MAGE gene expression and the disease stages were analyzed by the Mann-Whitney nonparametric U test.

Generation of Dendritic Cells. PBMCs were isolated from the heparinized venous blood of the patient from whom the FY cell line was established, by Ficoll-Hypaque (Lymphoprep; Nycomed Labs, Oslo, Norway) gradient centrifugation at 580 × g for 20 min and then washed with PBS three times. The adherent monocytes were cultured in RPMI 1640 supplemented with 5% human AB serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 μg/ml gentamicin, 2,000 units/ml recombinant IL-4 (R & D Systems, Inc., Minneapolis, MN), and 1,000 units/ml recombinant GM-CSF (R & D Systems, Inc.) for 7 days at 37°C. At the time of CTL induction in vitro, the DCs were pulsed with 10 μg/ml of the MAGE-3 epitope peptide for 4 h at 37°C in a serum-free medium, washed extensively, and then added to the bulk cultures of the PBMCs.

Phenotyping of DCs. The expressions of the cell surface antigens, MHC classes I and II, CD3, CD14, CD83, and CD86, were analyzed by flow cytometry using mAbs before and after the generation of DCs to evaluate the populations of the DCs generated from the PBMCs. The mAbs used were G46-2.6 (IgG1, anti-HLA-A, B, C), L243 (IgG2a, anti-HLA-DR; BD Biosciences, San Jose, CA), SK7 (IgG1, anti-CD3), SK3 (IgG1, anti-CD4), SK1 (IgG1, anti-CD8), MbP9 (IgG2b, anti-CD14); Becton Dickinson, San Jose, CA); HB-15e (IgG1, anti-CD83), and BU63 (IgG1, anti-CD86); Ancell, Bayport, MN). All of the data were analyzed using the CellQuest software (Becton Dickinson).

Synthetic Epitope Peptide. A nine amino acids-epitope with a sequence of IMPKAGLLI (amino acid position in MAGE-3, 195–203) was synthesized and provided by Takara Shuzo, Shiga, Japan. This MAGE-3 synthetic epitope peptide was evaluated previously and identified to bind to HLA-A24 molecules with a very high affinity (14).

Induction of CTL Responses. The PBMCs were collected from patient F. Y. and from HLA-A24+ healthy donors by the centrifugation of blood samples on a Ficoll-Hypaque density gradient as described above and cultured in RPMI 1640 containing 5% heat-inactivated human AB serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (Life Technologies, Inc.). As a stimulator, autologous DCs pulsed with MAGE-3 epitope peptide (PBMC:DC, 10:1) were added to the media every 7 days. Irradiated FY cells or nonpulsed autologous DCs were used as a control stimulator (PBMC:FY cells, 10:1). Recombinant IL-2 (R & D Systems, Inc.) was added to the media at a concentration of 300 units/ml every 3 days. The whole culture media was replaced on days 9 and 16. After 21 days of incubation, the cells were harvested, and the CTL activity was assessed. A flow cytometric analysis of the cell-surface antigens of the effector cells was performed. The cells were stained with mouse antihuman mAbs against CD3, CD4, and CD8 (Becton Dickinson). Isotype-matched mouse antibodies (Becton Dickinson) served as a negative control.

Cytotoxicity Assay. The target cells were labeled with 51Cr by incubating with 100 μCi of Na51CrO4 (New England Nuclear, Boston, MA) for 1 h at 37°C, washed four times, and plated onto round-bottomed, 96-well microtiter plates at a concentration of 5 × 103 cells/0.1 ml/well. Effector cells were then added at a concentration of 2.5 × 104 cells/0.1
Table 1  The expression of MAGE genes according to pathological stages in bladder and upper tract urothelial cancersa,b

<table>
<thead>
<tr>
<th>Pathological stage</th>
<th>No. of tumors</th>
<th>No. of MAGE + tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAGE-1</td>
<td>MAGE-2</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;pT1</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>pT2≤</td>
<td>11</td>
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<tr>
<td>Total</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>Upper tract urothelial cancer</td>
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<td></td>
</tr>
<tr>
<td>&lt;pT1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>pT2≤</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

a Clinical tissue samples were obtained from the patients at the time of surgery performed at Keio University Hospital.
b Expression of MAGE genes was detected by RT-PCR amplification of total RNA isolated from each sample using specific oligonucleotide primers.

Table 2  The expression of MAGE genes and HLA typing in established bladder cancer cell linesa,b

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pathology</th>
<th>MAGE-1</th>
<th>MAGE-2</th>
<th>MAGE-3</th>
<th>HLA-A2</th>
<th>HLA-A24</th>
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</thead>
<tbody>
<tr>
<td>KU-1</td>
<td>TCC, G2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>KU-7</td>
<td>TCC, G1</td>
<td>++</td>
<td>±</td>
<td>+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>KU-19-19</td>
<td>TCC, G3</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>T24</td>
<td>TCC, G2</td>
<td>–</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FY</td>
<td>TCC, G3</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

a Expression of MAGE genes was detected by RT-PCR amplification of total RNA isolated from cell lysates of each cell line using specific oligonucleotide primers.
b The relative levels of gene expression were determined as follows: the strongest positive band was defined as 100% and graded “+” for no expression; ±, <2% positive; +, 2–25% positive; ++, 26–50% positive; +++, >50% positive.

RESULTS

Expression of MAGE Genes in Bladder and Upper Tract Urothelial Cancers. We analyzed the expression of MAGE mRNA in the clinical tissue samples obtained at the time of the surgery (Table 1). A total of 9 of 28 (32%) bladder cancers expressed any of the three MAGE genes. The MAGE+ bladder cancer was significantly more frequent in muscle invasive disease (pT2≤) than in superficial disease (<pT1; 6%). In upper tract urothelial cancers, all of the invasive disease (pT2≤) expressed at least one of the MAGE genes, whereas two of four (50%) of the superficial diseases (<pT1) did; however, the difference was not statis-
tically significant. In the established human bladder cancer cell lines, all but KU-1 expressed MAGE genes (Table 2).

None of the samples that we examined, which were obtained from renal cell carcinoma and adrenal tumors, expressed MAGE genes, whereas in five testicular cancers, all of the samples were positive for at least one of the MAGE genes.3

**Dendritic Cell Preparation.** Remarkable changes in the cell surface markers of PBMCs obtained from patient F. Y. were observed with the in vitro 7-day stimulation by IL-4 and GM-CSF. In the single representative evaluation of cell surface markers by flow cytometry, a markedly increased expression of MHC class II (from 44% population of the whole PBMCs before the generation of DCs to 99% population of the adherent monocytes after a 7-day stimulation by IL-4 and GM-CSF), CD83 (from 4% to 96%), and CD86 (from 12% to 99%) was obtained, whereas a remarkable decrease was observed in the expression of CD3 (T-cell marker; from 57% to 1%) and CD14 (macrophage marker; from 18% to 1%). These changes in the surface markers of the populations of PBMCs indicated the successful generation of DCs.

**Induction of Autologous CTL Response against FY Cells by DCs Pulsed with HLA-A24-binding MAGE-3 Epitope Peptide.** Compared with the CTL response stimulated only by FY cells or nonpulsed autologous DCs, the cytotoxicity was significantly higher in the stimulation with autologous DCs pulsed with MAGE-3-encoded HLA-A24-binding peptide. The cytotoxic effect of this CTL was significantly blocked by the pretreatment of target FY cells with anti-HLA class I mAbs (Fig. 1A). In addition, the induced CTL did not recognize both KU-7 and KU-19-19, MAGE-3+, and HLA-A24− bladder cancer cells, but in contrast, moderately recognized T24, HLA-A24+, and MAGE-3 weakly positive bladder cancer cells (Fig. 1B). PBMCs were also collected from a HLA-A24+ healthy donor, and the effector cells were prepared according to the same method. These HLA-matched, but heter-
ologous, CTLs were also shown to respond to target FY cells as well as to autologous CTLs.

**Characterization of the Cytotoxic Effectors.** A flow cytometric analysis was performed during the induction of effector cells by DCs pulsed with the MAGE-3 epitope peptide, IMPKAGLLI (Fig. 2). The number of CD3+ cells gradually increased during the incubation. CD4+ cells decreased after 2 weeks, whereas the number of CD8+ cells kept increasing. CD3+ and CD8+ cells accounted for 98% and 66% of the effectors, respectively, after 3 weeks of induction.

**Clinical Cases.** A total of 4 HLA-A24+ patients who had metastatic MAGE-3+ bladder cancer with measurable lesions were treated in this pilot clinical trial (Table 3). These patients had already been treated intensively with surgery, chemotherapy, and radiotherapy. All of the bladder cancers resected from these patients were examined by RT-PCR and thus were proven to be MAGE-3+. None of the patients showed any unfavorable side effects throughout the DC vaccinations. A complete response of lymph node metastases was achieved in one patient, and a partial response was observed in two other patients. However, one patient died because of a progression of local recurrence and pleural dissemination. The first case (F. Y.) developed disseminated intravascular coagulation and died of sepsis attributable to a perforation of the small intestine 2 months after the DC vaccination; however, autopsy showed a complete remission of lymph node metastases, which were histologically evident before the DC vaccination. The second case (G. K.) showed the disappearance of a solitary liver metastasis and a >50% reduction in the size of the para-aortic lymph node metastasis evaluated by a computed tomography scan (Figs. 3 and 4). However, a solitary brain metastasis was apparent 6 months after the treatment, and was surgically removed. The third case (N. H.) showed a significant reduction in the size of inguinal lymph node metastases. A biopsy taken from the lymph node 3 months after the DC vaccination demonstrated significant necrotic changes in the lesions (Fig. 5).

**DISCUSSION**

*MAGE* genes are widely expressed in various cancers but not in normal tissues except for the testis, which makes *MAGE* potentially useful targets for tumor-specific immunotherapy. Patard et al. (18) reported that MAGE-1 and -3 were positive in 21% and 35% of tumors, respectively, in primary transitional cell carcinoma of the urinary bladder, and their expressions were more frequent in advanced stages of disease. In our current study, we first showed a high incidence of *MAGE* expression in bladder cancers and upper tract urothelial cancers. It was statistically significant and consistent with the findings of previous reports that the *MAGE* expression was observed more frequently as the tumor’s pathological stages advanced. Hence, the presence of *MAGE* in advanced urothelial cancers is highly expected. As a result, MAGE is increasingly suggested to be a useful target for active immunotherapy against bladder cancer. In this study, to establish the possibility of a modality of immunotherapy for invasive bladder cancers, we hypothesized that using autologous DCs pulsed with HLA-specific MAGE epitope peptide could induce autologous CTLs against *MAGE*-expressing bladder cancers.

DCs are well known to be professional antigen-presenting cells for the induction of a T cell-mediated immune response (33). High expression levels of adhesion molecules and costimulators, such as B7, on the cell surface and intracellular vesicles critical for antigen presentation are believed to prime CD4+ and CD8+ T cells (34, 35). Another means by which DCs induce potent T cell responses is via the release of IL-12. Using murine T cell receptor transgenic CD4+ T cells, Macatonia et al. (36) showed that DCs induced the differentiation of naive T cells into IFN-γ producing Th cells by stimulating them with IL-12 production. In addition, DCs induce potent human antiviral CD8+  

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4. T. Nishiyama, unpublished data.
CTL responses without any need for CD4+ T cells or exogenous cytokines (37). As these previous reports have indicated, DCs and macrophages have the ability to induce CTL and Th cell responses in vivo. DCs are shown to process exogenous antigens conventionally for presentation on MHC class II molecules. Bachmann et al. (38) reported that a cloned DC line is able to present cell debris-associated exogenous viral proteins to MHC class I-restricted CTLs in vitro. Before using DCs in anticancer immunotherapy, the epitope peptide that is most essential to anticancer CTL induction must be identified to avoid the risk of developing autoimmune diseases (13).

The antigenic peptides encoded by the MAGE-3 gene were shown to be presented by either HLA-A1 (39), -A2 (13), or -B44 (40) molecules (41), and Tanaka et al. (14) recently showed tumor-specific CTLs to be induced from healthy donors’ PBMCs by stimulation with HLA-A24-binding MAGE-3-derived synthetic peptide, IMPKAGLLI. HLA-A24 is positive in ~61% of the Japanese population (42). As a result, it makes sense to develop current DC-based immunotherapy using this specific synthetic epitope peptide.

In our current experiment, we have shown the successful induction of the CTL response by stimulating PBMCs with autologous DCs pulsed with a HLA-A24-binding MAGE-3 epitope peptide. This CTL was considered to recognize MAGE-3+ tumor cells in a MHC class I-restricted manner because the cytotoxic effect of this CTL against FY cells was significantly blocked by the pretreatment of FY cells with anti-HLA class I mAbs. In addition, the induced CTL did not recognize either KU-7 or KU-19-19, both of which are MAGE-3+ but HLA-A24− bladder cancer cells. On the other hand, CTL activity against FY cells was seen in the effector cells prepared from a nonrelated healthy donor with HLA-A24 typing, thus indicating that the basic mechanism underlying this modality requires matching for both HLA typing and tumor-specific peptide, which is expressed in target cells and is also presented to effector cells by DCs in vitro. These achievements using in vitro experiments formed the basis for conducting clinical trials with this DC-based immunotherapy in patients with advanced bladder cancers and histologically proven, measurable metastatic lesions. Our initial findings of clinical trials with only the four cases presented herein indicated that tumor-specific, DC-based cancer immunotherapy may be useful as a new additional treatment modality for advanced bladder cancer that is not curable by surgery, chemotherapy, or radiotherapy.

Numerous concerns still exist regarding the application of DCs in clinical treatment. Most of the patients with progressive cancers are likely to be of advanced age and to have a poor immunological status. The combination therapies with DCs and the systemic administration of cytokines are possible solutions. IL-12 is known to induce CTLs in patients with a poor immune

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**Fig. 3** Complete response of a metastatic lesion in liver by MAGE peptide-pulsed DC immunotherapy in a patient with advanced bladder cancer. CT scan shows a solitary liver metastatic lesion (arrow in Fig. 3A) completely disappeared after 18 × biweekly DC vaccination (B).

**Fig. 4** Effectiveness of the MAGE peptide-pulsed DC immunotherapy against paraaortic lymph node metastasis of bladder cancer. A bulky paraaortic lymph node metastasis was observed by computed tomography scan before the MAGE peptide-pulsed DC vaccination (A; arrow) in the same patient as in Fig. 3. A >50% reduction in the size of metastasis was evident after the DC vaccination (B, arrow).
status, and the concomitant use of IL-12 may reduce the need for large doses of DCs (43). In our preliminary experiments in vitro, when IL-12 was used to stimulate effector cells from PBMCs along with the autologous DCs pulsed with MAGE-3-encoded HLA-A24-binding epitope peptide, the CTL activity was significantly higher than the effector cells prepared without IL-12.5 Additional studies based on these recent findings are now under way. Such studies should focus on the safety and the feasibility of using this DC-based active immunotherapy against advanced bladder cancers.

In summary, autologous DCs pulsed with HLA-A24-binding MAGE-3 epitope peptide have been shown to successfully induce MHC class I-restricted MAGE-3 specific autologous CTLs in vitro in MAGE-3+ bladder cancer. The efficacy of DCs pulsed with the epitope peptide remains to be elucidated, because its clinical application has just been initiated. However, our results clearly showed a good potential for the development

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5 T. Nishiyama, unpublished data.

Fig. 5 Histopathological evaluation of representative biopsy specimens taken from inguinal lymph node metastasis of a bladder cancer patient before and after the MAGE peptide-pulsed DC immunotherapy. A typical transitional cell carcinoma was evident before the DC vaccination (A). Significant necrotic changes (arrows) were demonstrated 3 months after the DC vaccination (B; H&E, original magnification ×100).
of a DC-based, tumor-specific immunotherapy for the treatment of bladder cancer.

REFERENCES


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