SPANDIDOS PUBLICATIONS

Potential role of dendritic cell vaccination with MAGE peptides in gastrointestinal carcinomas

FUMIAKI TANAKA, NAOTSUGU HARAGUCHI, KENJI ISIKAWA, HIROSHI INOUE and MASAKI MORI

Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu 874-0838, Japan

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Abstract. Dendritic cells (DCs) loaded with tumor antigens have been emerging as a new strategy in cancer treatment. The *MAGE* genes are selectively expressed in a variety of cancer tissues such as melanoma or gastrointestinal carcinomas. However, no expression is observed in normal tissues except testis. There are several reports on clinical trials with these immunogenic peptides including MAGE gene-derived, which were shown to be effective for some patients with carcinomas. We previously reported a clinical trial treating gastrointestinal carcinoma patients with immature DC and MAGE peptides via intravenous injection. Autologous DCs were generated *ex vivo* and were pulsed with MAGE-3 peptide, depending on the patient's HLA haplotype (HLA-A02 or A24). In this study, patients were immunized with mature DCs pulsed with the MAGE-3 peptide four times every 2 weeks via s.c. injection close to the axilla and inguinal lymph nodes. Twenty-eight patients with advanced gastrointestinal carcinoma were treated and no toxic side effects were observed. Peptide-specific CTL responses, improvement in performance status, tumor marker decrease and minor tumor regressions after vaccination were observed in some patients. These results suggested that DC vaccination with the MAGE-3 peptide would be safe and can exhibit antitumor effects even in the patients with advanced gastrointestinal carcinoma who were previously treated with chemotherapy or radiation therapy.

Introduction

New therapeutic strategies are required for many inoperable and recurrent cancers due to the lack of effective treatment. Gastrointestinal cancers are common malignant tumors and are also a major cause of cancer-related death in the world (1,2). Most patients who undergo surgery for advanced stage gastrointestinal carcinomas are still at high risk of local or systemic recurrence. Recently, chemotherapy has been improved for treatment of patients with advanced colorectal cancer. However, there is no standard therapy including chemotherapy or radiotherapy for advanced or recurrent variety of gastrointestinal cancers. Thus, there is a great need for novel therapeutic approaches for patients with advanced or recurrent gastrointestinal carcinomas.

The expression of the *MAGE* genes was observed in tumors of various histological origins, whereas no expression has been observed in normal tissues except testis (3). Several reports exist on the successful induction of HLA class I-restricted antitumor CTLs using MAGE peptides (4-8). These tumor antigen-peptides seem to be potential targets for tumor-specific immunotherapy (9-11).

Dendritic cells (DCs), antigen-presenting cells, which were the most capable of priming naive T cells to CTLs, have been demonstrated to induce potent antitumor immunity *in vitro* and *in vivo* (12). By using the efficacy of DCs, clinical trials using DCs have been studied as an active immunotherapy against a variety of carcinoma including malignant melanoma. DC vaccination induced tumor-specific immune responses and also tumor regression in clinical trials for several types of carcinomas (13-15).

We previously reported DC vaccination with HLA-restricted MAGE-3 peptide for patients with gastrointestinal carcinoma in twelve patients (16). No toxicity was found in any patient and the immune response for MAGE-3 peptide and tumor regression was observed in some patients who had advanced metastatic gastrointestinal carcinoma. Based on our previous study, we continued the DC vaccination with HLA-restricted MAGE-3 or MAGE-1 peptides in 28 patients with gastrointestinal carcinoma. This study summarizes the results; no toxicity was found in any patient and the immune response for MAGE peptide and tumor regression was observed in some patients who had advanced metastatic gastrointestinal carcinoma.

Patients and methods

Patients. The study protocol was approved by the Clinic Institutional Ethics Review Boards of the Medical Institute of Bioregulation, Kyushu University and written consent was obtained from all the patients at the time of enrollment.

Correspondence to: Professor Masaki Mori, Department of Surgery, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874-0838, Japan
E-mail: mmori@beppu.kyushu-u.ac.jp

Abbreviations: DC, dendritic cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; SCC, squamous cell carcinoma antigen; PD, progressive disease; MR, minor response; DTH, delayed type hypersensitivity; Th, helper T cells

Key words: dendritic cell, MAGE peptides, gastrointestinal carcinomas

According to the protocol, patients were required: a) to be HLA-A2 (0201) or HLA-A24 (2402)-positive; b) to have histologically confirmed primary or metastatic lesions of gastrointestinal carcinoma expressing *MAGE-3* mRNA by reverse transcription-PCR (17); c) to have adequate cardiac, pulmonary, hepatic, renal and hematological functions; and d) to have an ECOG performance status of 0 to 2. Furthermore, patients were excluded a) with any severe infectious, hematological, cardiac and pulmonary diseases; b) with radiation therapy, chemotherapy, or immunotherapy within the prior 4 weeks; c) with steroid therapy; and d) in pregnancy. Treatment was carried out at the Department of Surgery, Medical Institute of Bioregulation, Kyushu University from January 1997 through August 2006.

Generation of DCs. Patients underwent leukapheresis using a cell separator (MULTI; Hemonetics Co., Braintree, MA). PBMCs isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) were separated by adherence to a plastic tissue culture flask to enrich the monocyte fraction. After 90 min at 37˚C, nonadherent cells were removed and adherent cells were subsequently cultured for 7 days with 1000 U/ml of granulocyte macrophage colony-stimulating factor (NCPC-GeneTech, Shijiazhuang, P.R. China) and 1000 U/ml of IL-4 (R&D system, Minneapolis, MN) in RPMI with 5% autologous serum. On day 6, OK432 (0.1 KE/ml) (Chugai Co., Osaka, Japan) was added to the culture medium for 2 days to induce maturation of the DCs. After 7 days, the DCs were harvested by vigorous washing from the flask and the remaining cells were removed with cell dissociation buffer (Life Technologies, Inc., Gaithersburg, MD). Cultured DCs were monitored by light microscopy. Flow cytometric analysis was performed using a FACScan (Becton Dickinson) with antibodies against mouse antihuman HLA-class I (Immunotech, Marseille, France), HLA-DR (Immunotech), CD80 (Ancell, Bayport, MN), CD86 (Ancell), or CD14 (Becton Dickinson, San Jose, CA). FITC-conjugated rabbit antimouse IgG was used as the second antibody (Dako Japan Co., Ltd., Kyoto, Japan).

Pulsing of in vitro generated DCs. Generated DCs were resuspended at $1x10^6$ cells/ml normal saline with 1% human albumin. DCs were pulsed with 10 μg/ml of MAGE-3 peptide for HLA-A2 (FLWGPRALV) (6) and for HLA-A24 (IMPKAGLLI) (8), was synthesized and purified (>95% purity) by Mutiple Peptide System (San Diego, CA) for 4 h at room temperature.

Patient treatment. The standard vaccination schedules were as follows. Four vaccinations with MAGE-3 peptide-pulsed DCs were administered at 14-day intervals. Seven days before each vaccination, patients underwent leukapheresis for DC set up. Physical and hematological examinations were monitored before and after each vaccination. The DC vaccines were immunized with $3x10^7$ cells (pulsed with 300 μg of peptide)/1.6 ml normal saline. The patients were to receive the DC s.c. every 2 weeks for four immunizations (Fig. 1). Toxicity was graded using the National Cancer Institute-Common Toxicity Criteria. Tumor markers [carcinoembryonic antigen (CEA), CA19-9 and SCC] and imaging studies (computed tomography scans and chest radiographs) were reviewed as available before, during and after the four immunization protocols to determine the clinical response. Standard definitions of major objective responses (complete response, partial response, no change, or PD) were used. MR was defined as a 25 to 50% decrease of lesions in at least 1 month or a >50% decrease of lesions lasting less than a month. Performance status was re-evaluated at the end of treatment according to the ECOG scale.

Immunological response. Blood samples were collected for assessment of CTL precursors from the first leukapheresis and the fourth leukapheresis and PBMCs were separated by centrifugation on a Ficoll-Paque density gradient. PBMC preparations were frozen in FCS with 10% DMSO. CTL assay was performed according to the protocol, as described (18). Briefly, $4x10^6$ PBMCs/ml were incubated in RPMI-1640 with 5% heat-inactivated human serum in 24-well plates in the presence of 20 μg/ml MAGE-3 peptide. On day 1, recombinant interleukin-2 (Takeda Co., Ltd., Osaka, Japan) was added to the culture at 30 IU/ml. On day 7, cells were centrifuged and resuspended at $5x10^5$ cells/ml in the presence of $1x10^6$ cells/ml peptide-pulsed PBMCs and 30 IU/ml IL-2 was added on day 8. Peptide-pulsed PBMCs were pretreated with mitomycin C (Kyowa Hakko, Osaka, Japan). The CTL activities were tested on day 14. The target peptide-pulsed cell lines, 221(A2.1) [HLA-A2 (+), MAGE-3 (-)] for HLA-A2 and TISI [HLA-A24 (+), MAGE-3 (-)] for HLA-A24 (both cell lines were provided by Takara Shuzo Co. Ltd.) were prepared by incubating the cells with the peptides (20 μg/ml) overnight at 37˚C. The target cancer cell lines, the gastric carcinoma cell line KATO III [HLA-A2(+), -A24 (-), MAGE-3 (+)], the colon carcinoma cell line WiDr [HLA-A24(+), MAGE-3(+)] and the lymphoma cell line Raji [HLA-A2(-), A24(-), MAGE-3 (-)] were provided Japanese Cancer Research Bank (Tokyo, Japan). These cells were labeled with 100 μCi of sodium 51 chromate (^{51}Cr) for 1 h at 37˚C and the labeled cells were then washed and resuspended. The effecter cells were placed in each well of round-bottomed microtiter plates. The labeled target cells were then added to the well at a concentration of $3x10^3$ cells/well to produce a total volume of 0.2 ml. After a 4-h incubation period, the release of ^{51}Cr label was measured by collecting the supernatant, followed by quantitation in an automated gamma counter. The percentage of specific cytotoxicity was calculated as the percentage of specific ^{51}Cr release: 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release). To eliminate any nonspecific lysis attributable to natural killer-like effectors, the cytolytic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells.

DTH skin tests were performed with peptide before vaccination and after the four-immunization protocol. Patients were injected with 10 μg of peptide (100-μl final volume) intradermally at disease-free sites. A positive skin test reaction was defined as palpable skin indurations of at least 4 mm in diameter combined with erythema of at least the same size at the site of peptide inoculation after 48 h (19).

Flow cytometric determination of IFN and IL-4 in the cytoplasm of peripheral CD4-positive T cells was performed, as described (20). Briefly, the patient's CD4-positive T cells

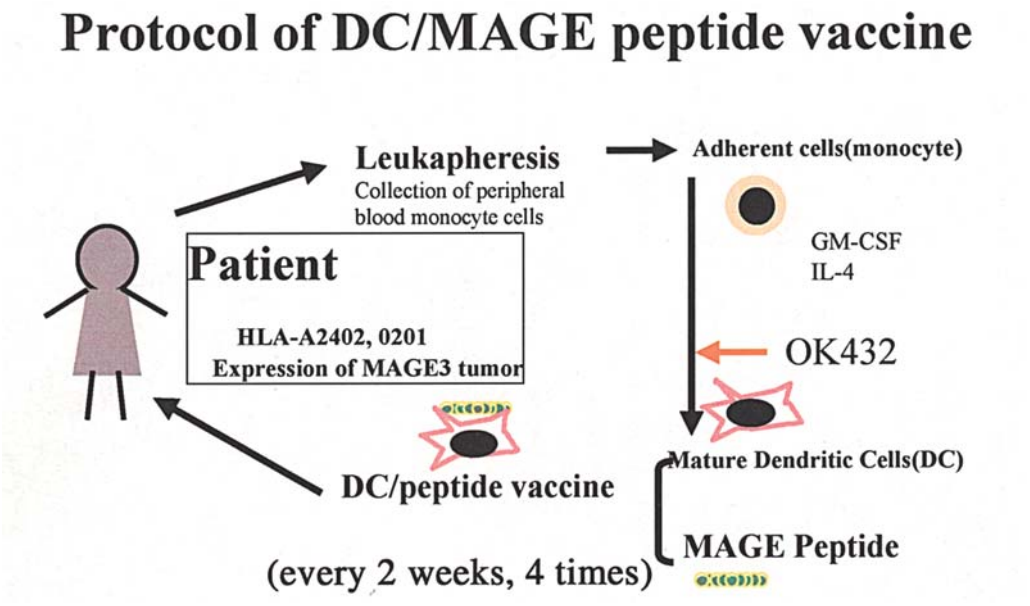

Figure 1. Protocol of DC/MAGE peptide vaccine. When a patient has expression of MAGE and HLA-A2402 or 0201, peripheral monocyte cells were collected and dendritic cells were generated using GM-CSF, IL-4 and OK432. MAGE peptide and DCs were co-cultured with DC, then the DC/peptide was injected into axiller and inguinal lymh nodes. This vaccine was repeated every 2 weeks and completed with injection (4 times).

were continuously treated with fluorescence-activated cell sorter lysing and permeabilization solutions (Becton Dickinson Immunocytometry System; Becton Dickinson). The cells were subsequently incubated with FITC-conjugated anti-IFN- and phycoerythrin-conjugated anti-IL-4 (Becton Dickinson) in 0.1% BSA-PBS. FITC-mouse IgG_2a and phycoerythrin-mouse IgG_1 (Becton Dickinson) were used as controls. The percentage of cells positive for IFN and IL-4 were counted and evaluated with a FACScan (Becton Dickinson).

Immunohistochemistry. Serial paraffin-embedded tissue sections of carcinoma tissues were stained with monoclonal antibodies against MAGE-3 (57B; kindly provided by Dr Giulio Spagnoli, University Hospital, Basel, Switzerland), T cells (UCHL-1; Dako), CD8 (C8/144B; Nichirei Co., Tokyo, Japan), or CD4 (1F6; Nichirei Co.). Primary antibody was detected with Dako LSAB kit, Peroxidase (Dako). Diaminobenzidine tetrahydrochloride was used as the chromogen. Then, the sections were counterstained with hematoxylin.

Results

Patients. The characteristics of the 28 patients initially enrolled in the study are summarized in Table I. All of the patients had MAGE-3-expressing advanced gastrointestinal carcinomas originating from the stomach (ten patients), esophagus (eight patients), colon (seven patients), gall bladder (two patients) and pancreas (one patient).

DCs. The collected PBMCs were $1.31\pm0.42\text{x}10^9$ cells after each leukapheresis and Ficoll separation. To generate DCs from PBMCs, $15.51\pm0.5\text{x}10^7$ PBMCs were plated and after 7-day culture, $3.51\pm0.49\text{x}10^7$ cells were obtained with 95% viability. By morphology, the harvested population of cells was 72±13% large dendritic-like cells and 18±8% small lymphoid-like cells. DCs expressed high levels of HLA class I, class II, CD80, CD86 and low CD14 by flow cytometry (data not shown).

Toxicity. The vaccination protocols were well tolerated. There were no acute toxicities during or immediately after the s.c. DC injection. No hematological, hepatic, pulmonary, or renal toxicities were observed in any patients (Table I).

Immunological response. Aliquots of PBMCs, frozen at the first leukapheresis and the fourth leukapheresis, were thawed at the same time and subjected to the assay for CTL precursors. We evaluated the CTL response for eight patients. A CTL response was considered to be positive with a peptide-specific lytic activity >20% at an E:T ratio of 80:1 or 40:1. Results from our preliminary test series indicated that a target lysis of 20% (E:T, 80:1 or 40:1) was a suitable cutoff to evaluate the peptide-specific lysis (8). Before vaccination, CTL precursor frequencies were low or undetectable. On the other hand, the peptide-specific CTL responses were observed in four out of eight patients after vaccination (Fig. 2).

Peptide-specific DTH reactions were not observed before vaccination in any patient; however, there were eight patients in which we observed DTH reactions after the fourth vaccination (Table I). Intracellular cytokine analysis was performed for twenty-two patients. In eight of the 22 patients, the ratio of IFN-γ:IL-4 of CD4-positive cells increased after vaccination compared with before vaccination (Table I).

Clinical response. Table I summarizes the clinical response for the 28 patients. In eleven patients, tumor markers (CEA, CA19-9 or SCC) decreased after the first or second vaccination compared with before treatment. Regression of tumor was observed in four patients. In one case, metastatic

Table I. Patient records.

Case	Primary site	Metastasis	Pre-treat	Side effect	Tumor marker	Antitumor effect	Better QOL	Th1/Th2
1	Esophagus	Neck LN	S,C,R	No	Down	MR	Yes	Up
2	Esophagus	Neck LN	C,R	No	Up	PD	NC	Down
3	Esophagus	Abd LN	C,R	No	Down	MR	Yes	N.D.
4	Stomach	Peritoneum	S,C	No	Down	PD	Yes	N.D.
5	Stomach	Peritoneum, liver	S,C	No	Up	PD	No	N.D.
6	Stomach	Peritonuem, wall	S,C	No	Down	PD	No	N.D.
7	Stomach	Lung	S,C	No	Down	PD	No	N.D.
8	Stomach	Liver	S,C	No	Down	PD	NC	N.D.
9	Stomach	Liver, abd wall	S,C	No	Up	PD	No	Down
10	Colorectal	Lung, chast wall	S,C	No	Down	MR	Yes	Up
11	Colorectal	Liver	S,C	No	Up	PD	NC	Down
12	Colorectal	Bone	S,C	No	Up	PD	NC	Up
13	Stomach	Abd LN	S,C	No	Up	PD	No	Down
14	Stomach	Bone	S,C	No	Down	PD	No	Down
15	Stomach	Peritoneum	S,C	No	Up	PD	No	Down
16	Colorectal	Liver, abd LN	S,C	No	Down	NC	NC	Dp
17	Gall Bladder	Lung, abd LN	S,C	No	Down	MR	NC	Up
18	Colorectal	Liver, abd LN	S,C	No	Up	PD	No	Down
19	Stomach	Abd LN	S,C	No	Up	PD	No	Down
20	Esophagus	Neck LN	S,C,R	No	Up	PD	No	Down
21	Esophagus	Liver	S,C	No	Up	PD	NC	Up
22	Colorectal	Local, abd LN	S,C	No	Up	PD	No	Down
23	Pancreas	Liver, local	S,C	No	Up	PD	NC	Up
24	Esophagus	Liver	C,R	No	Down	PD	No	Down
25	Gall Bladder	Liver, abd wall	S,C	No	Up	PD	NC	Down
26	Colorectal	Abd LN	S,C,R	No	Up	PD	Yes	Up
27	Esophagus	Chest	S,C	No	Up	PD	NC	Down
28	Esophagus	Chest	S,C,R	No	NC	PD	Yes	Down

LN, lymph node; Abd, abdominal; S, surgery; C, chemotherapy; R, radiation therapy and NC, no change.

lesions of supraclavicular lymph nodes regressed after the second vaccine and also the hoarseness was improved. In another case, metastatic lymph nodes that were resistant to prior chemotherapy and irradiation regressed and esophagectomy was performed after the DC vaccine protocol. In another case, atelectasis of the left lung improved. Six patients showed an improvement in performance status and four patients maintained the performance status compared with their status pretreatment. Twenty-four out of 28 patients felt some pain at the site of DCs/peptide complex on injection day. However, all patients recovered the following day.

Discussion

In this study, the mature DC vaccines with MAGE peptide injection were well tolerated with low toxicity. The results were consistent with our previous study and others (21-24). As we performed a dose escalation study in our previous study, we fixed the vaccine-cell number (30 million DCs/injection).

Gastrointestinal carcinomas, such as esophageal, gastric and colorectal carcinomas may be curable by surgery, though the cure rate is moderate to poor depending on the extent of the disease. Regarding gall bladder carcinoma and pancreatic cancer, it is very difficult to treat them by surgery alone (25,26). Chemotherapy may have some benefit especially for colorectal cancer. However, there is no standard regimen for advanced gastrointestinal carcinomas, especially for upper gastrointestinal cancer. Adverse events sometimes occur. Thus, cancer immunotherapy for gastrointestinal carcinomas could be an alternative treatment approach. Compared to our previous study, we changed these points; i) use of mature DCs from immature DCs and ii) i.v. injection to s.c. injection. To protect generating auto-immune reaction, and avoiding the state of anergy to tumor immunity, using mature DCs is an important factor (27). Injection of s.c. would be more effective and available for trafficking to 'regional lymph node' than that of i.v. injection. These changes might be reflected in the increase in percentage of DTH-positive patients.

The expression of the *MAGE* genes was observed in tumors of various histological origins, whereas no expression has been observed in normal tissues except testis (3). Testis cells do not express MHC class I molecules, thus, they do

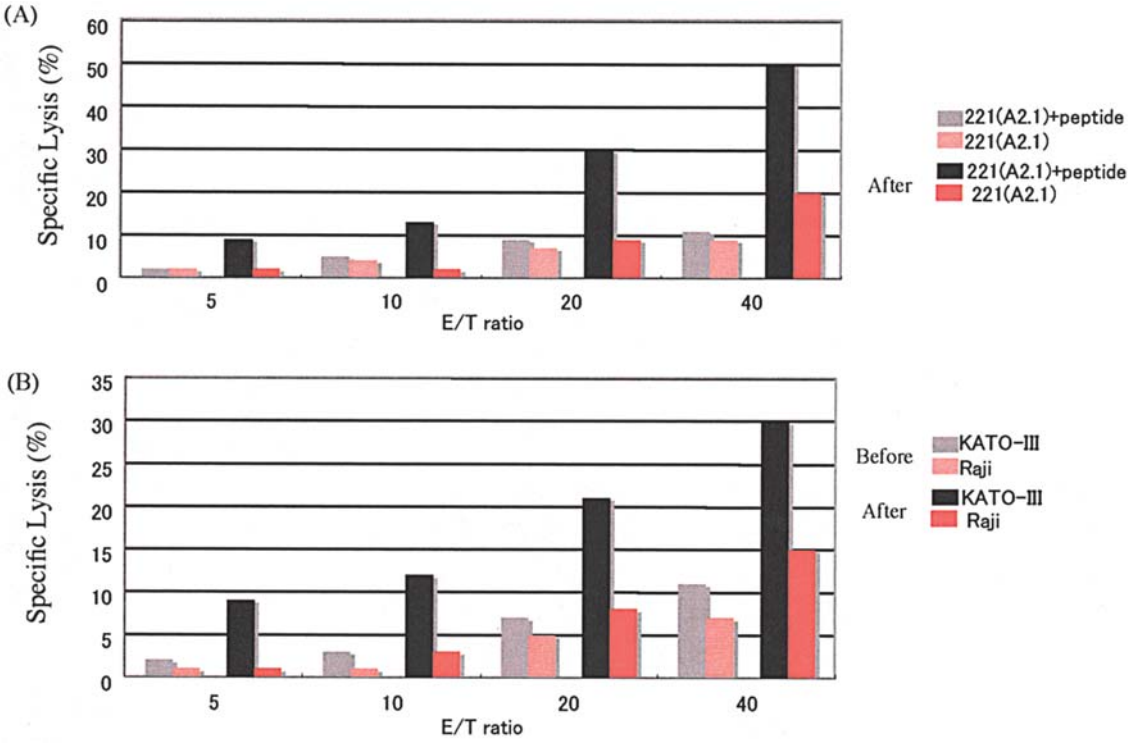

Figure 2. DCs/MAGE peptide vaccine enhanced immunological response against cells expressing MAGE-3 and HLA-A0201. PBMCs were obtained from patients before the first vaccination and after the final injection. (A) Cytolytic assay was performed against 221(A2.1) cells alone and 221(A2.1) cells pulsed with the MAGE peptide. (B) Cytolytic assay was performed against KATO-III cells (HLA-A0201-positive and MAGE-3-positive) and Raji cells (HLA-A0201-negative and MAGE-3-negative).

not present MAGE peptides at their surface. Therefore, immunotherapy with MAGE-derived antigens could avoid unexpected diseases such as autoimmune disease. Previous studies including ours indicated that no significant toxicity was observed in clinical trials with the MAGE-3 peptide for our gastrointestinal, melanoma patients and urinary bladder cancer patients (15,16,28). This study also found no significant toxicity; therefore, the MAGE peptide is consistently considered as a good target for gastrointestinal carcinomas.

Based on our study, DCs pulsed with HLA-restricted peptides is also applicable for gastrointestinal carcinomas because of the general safety and feasibility of this approach, in addition to demonstrating an immunological and clinical response for several tumor types.

Mainly Th1 cells produce IFN-γ and mediate cellular immune response, whereas Th2 cells produce mainly IL-4 and mediate humoral responses (29). Among T cells from PBMC in advanced gastrointestinal cancer patients, a Th2-subset dominance was shown (30). In the present study, we observed the ratio of IFN-γ/IL-4 of CD4-positive cells (Th1/Th2) increased after vaccination in some patients who had regression of tumor. These results indicate that a systemic cellular immune response may be induced in addition to a tumor-specific immune response in effective cases of DC vaccination. However, the increase of Th1/Th2 did not include all the patients who showed clinical benefits such as tumor marker decrease and/or tumor shrinkage. Further analysis is required to evaluate and predict the clinical benefits from immunological response. We performed some ELISPOT assays using PBMCs from these patients, however, the results indicate that the ELISPOT assay was not adequate to show a sensitive or specific effector response to the MAGE peptides (data not shown).

In the present study, peptide-specific immune responses were recognized in some patients by *in vitro* CTL precursor assay and DTH response. However, there is no direct evidence of a correlation between the immune response and clinical tumor regression. In the future, consideration should be given to this type of immunotherapy with other treatment strategies to achieve a greater vaccine effect. To avoid the negative effect on DC vaccine mediated by $CD4^+/CD25^+$ Tregs, depletion of them using a recombinant IL-2 diphtheria toxin conjugate DAB_{389}IL-2 in human cancer patients (31), or murine model (32) was reported to be effective. We are planning the next clinical trial to assess whether vaccine therapy may become another modality for adjuvant treatment of gastrointestinal carcinomas at high risk for recurrence after operation, such as combination with or without chemotherapy or radiotherapy. To discover new markers to predict the good candidate for immunotherapy is crucial, because this approach could achieve clinical benefits for patients with advanced gastrointestinal cancer.

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