

Dendritic Cell Vaccination with MAGE Peptide Is A Novel Therapeutic Approach for Gastrointestinal Carcinomas¹

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ABSTRACT

The *MAGE* gene is selectively expressed in cancer tissues such as melanoma or gastrointestinal carcinomas, whereas no expression is observed in normal tissues except testis. There are several reports of successful induction of HLA class I-restricted antitumor CTLs using MAGE peptides, and some clinical trials with these immunogenic peptides were reported as effective for some patients with malignant melanoma. However, there are no similar studies in gastrointestinal carcinomas, which are important neoplasms. Autologous dendritic cells (DCs) were generated *ex vivo* and were pulsed with MAGE-3 peptide, depending on the patient's HLA haplotype (HLA-A2 or A24). Patients were immunized with DC pulsed with MAGE-3 peptide every 3 weeks at four times. Twelve patients with advanced gastrointestinal carcinoma (six stomach, three esophagus, and three colon) were treated, and no toxic side effects were observed. Peptide-specific CTL responses after vaccination were observed in four of eight patients. Improvement in performance status was recognized in four patients. Tumor markers decreased in seven patients. In addition, minor tumor regressions evidenced by imaging studies were seen in three patients. These results suggested that DC vaccination with MAGE-3 peptide is a safe and promising approach in the treatment of gastrointestinal carcinomas.

INTRODUCTION

Gastrointestinal carcinomas are quite common malignant tumors and also a major cause of cancer-related death in the world (1). It is well recognized that most patients who undergo operation for gastrointestinal carcinomas remain at high risk for local or systemic relapse. However, there is no standard therapy including chemotherapy or radiotherapy for advanced or recurrent tumors. Thus, there is a great need for novel therapeutic approaches for patients with advanced or recurrent gastrointestinal carcinomas.

The *MAGE* genes are expressed in a significant proportion of malignant tumors of various histological origins, whereas no expression has been observed in normal tissues except testis (2). There are several reports of successful induction of HLA class I-restricted antitumor CTLs using MAGE peptides (3-7). These tumor antigens seem to be potential targets for tumor-specific immunotherapy. Clinical trials involving the immunization of cancer patients using MAGE peptide have already started (8, 9), and tumor regression was reported in some metastatic melanoma patients (10).

DCs³, antigen-presenting cells capable of priming naive T cells to specific antigens in an HLA-restricted fashion, have been demonstrated to induce potent antitumor immunity *in vitro* and *in vivo* (11). On the basis of these understandings, clinical trials using DCs have been studied as an active immunotherapy especially for malignant melanoma. DC vaccination induced tumor-specific immune responses and also tumor regression in clinical trials for malignant melanoma (12, 13).

We reported previously that the expression rate of *MAGE-3* mRNA is relatively high in samples of gastrointestinal carcinomas [57% of esophageal carcinomas (14); 38% of gastric carcinomas (15); 19% of colorectal carcinomas (16); and 68% of hepatocellular carcinomas (17)]. In addition, we have identified recently (7) an HLA-A24-restricted MAGE-3 peptide because HLA-A24 is the most common HLA class I allele and HLA-A2 is the second most common allele in the Japanese population (present in 61% and 44%, respectively). In the present study, we applied DC vaccination for HLA-A24 or -A2-positive patients with MAGE-3 expressing advanced gastrointestinal carcinomas. To our knowledge, this is the first report of DC vaccination with HLA-restricted MAGE-3 peptide for patients with gastrointestinal carcinoma. No toxicity was found in any patients, and the immune response for MAGE-3 peptide and tumor regression

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³ The abbreviations used are: 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; ECOG, Eastern Cooperative Oncology Group.

was observed in some patients who had advanced metastatic gastrointestinal carcinoma.

MATERIALS AND METHODS

Patients. The study protocol had been approved by the Clinic Institutional Ethical Review Boards of Medical Institute of Bioregulation, Kyushu University, and written consent was obtained from all of the patients at the time of enrollment. According to the protocol, patients were required: (a) to be HLA-A2 (0201, 0206) 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 (15); (c) to have adequate cardiac, pulmonary, hepatic, renal, and hematological function; and (d) to have an ECOG performance status of 0 to 2. Furthermore, patients were excluded (a) with any severe infectious, hematological, cardiac, pulmonary disease; (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 2000.

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 units/ml of granulocyte macrophage colony-stimulating factor (kindly provided by Schering-Plough Co., Madison, NJ) and 1000 units/ml of IL-4 (Schering-Plough Co.) in RPMI with 5% autologous serum. 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 (Ansell, Bayport, MN), CD86 (Ansell), 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 1×10^6 cells/ml normal saline with 1% human albumin. DCs were pulsed with 10 µg/ml of MAGE-3 peptide for HLA-A2 [FLWGPRALV (5) was synthesized and purified (>95% purity) by Bachem AG (Bubendorf, Switzerland)] and for HLA-A24 [IMPKAGLLI (7) was synthesized and purified (>95% purity) by Takara Shuzo Co., Ltd. (Otsu, Japan)] for 4 h at room temperature.

Patient Treatment. The standard vaccination schedules were as follows. Four vaccinations with MAGE-3 peptide-pulsed DCs were given at 21-day intervals. Seven days before each vaccination, patients underwent leukapheresis for DC set up. Physical examinations and hematological examinations were monitored before and after each vaccination. The first DC vaccine was immunized with 1×10^7 cells (pulsed with 100 µg

of peptide)/10 ml normal saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), and the second, third, and fourth vaccines were immunized with 3×10^7 cells (pulsed with 300 µg of peptide)/30 ml normal saline. The patients were to receive the DC i.v. over 3 min every 3 weeks for four immunizations. Toxicity was graded using the National Cancer Institute-Common Toxicity Criteria. Tumor markers (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 more than 50% decrease of lesions lasting less than a month. Performance status was reevaluated 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, 4×10^6 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 5×10^5 cells/ml in the presence of 1×10^6 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 effector 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 3×10^3 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: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. 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

Table 1 Patients enrolled in the study

Case ^a	Age	Sex	HLA	Primary	Metastasis ^b	Previous treatment ^c
1	50	Male	A2	Stomach	Peritoneum	S, C
2	68	Male	A2	Stomach	Peritoneum, liver	S
3	73	Male	A2	Stomach	Peritoneum, abdominal wall	S, C
4	78	Female	A24	Stomach	Lung, lymph nodes (mediastinum)	S, C
5	80	Male	A24	Stomach	Liver	S
6	67	Female	A24	Stomach	Liver, peritoneum	S, C
7	62	Male	A2	Esophagus	LN (mediastinum, abdomen)	S, C
8	60	Male	A2	Esophagus	LN (abdomen), pancreas	C, R
9	81	Male	A24	Esophagus	LN (neck)	R
10	57	Male	A2	Colon	Lung, chest wall	S, C
11	59	Male	A24	Colon	Liver	S, C
12	57	Female	A24	Colon	Bone	S, C

^a Withdrawn after second vaccine (cases 2, 4, and 6).

^b LN, lymph nodes.

^c S, surgery; C, chemotherapy; R, radiotherapy.

defined as a palpable skin induration 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- γ 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 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- γ and phycoerythrin-conjugated anti-IL-4 (Becton Dickinson) in 0.1% BSA-PBS. FITC-mouse IgG_{2a} and phycoerythrin-mouse IgG₁ (Becton Dickinson) were used as controls. The percentage of cells positive for IFN- γ 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; Ref. 21), 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. Finally, the sections were counterstained with hematoxylin.

RESULTS

Patient Characteristics. The characteristics of the 12 patients initially enrolled in the study are summarized in Table 1. There were nine men and three women with a median age of 66 years (range, 50–81). All of the patients had MAGE-3-expressing advanced gastrointestinal carcinomas originating from the stomach (six patients), esophagus (three patients), and colon (three patients). Three patients succumbed to cancer after two vaccinations (cases 2, 5, and 7). Nine patients received all of the four planned vaccine protocols.

DCs. The collected PBMCs were $1.01 \pm 0.36 \times 10^9$ cells after each leukapheresis and Ficoll separation. For the first vaccine, $8.93 \pm 0.92 \times 10^7$ PBMCs were plated, and after 7-day culture, $1.36 \pm 0.36 \times 10^7$ cells were obtained with 90% viability. For the second, third, and fourth vaccine, $16.83 \pm 0.1 \times 10^7$ PBMCs were plated, and after 7-day culture, $3.34 \pm$

Table 2 Response to DC vaccine therapy (I)

Case ^a	Toxicities	CTL		
		precursor	DTH ^b	IFN- γ /IL-4 of CD4
1	–	–	–(0)	ND ^c
2	–	ND	ND	ND
3	–	+	ND	ND
4	–	ND	ND	ND
5	–	+	+(8)	ND
6	–	ND	ND	Decrease (15.4 \rightarrow 7.3)
7	–	+	+(5)	Decrease (13.8 \rightarrow 12.9)
8	–	–	–(2)	Increase (2.9 \rightarrow 5.3)
9	–	+	–(0)	ND
10	–	–	+(5)	Increase (8.2 \rightarrow 10.8)
11	–	–	–(0)	Decrease (8.4 \rightarrow 6.3)
12	–	ND	–(2)	Increase (5.3 \rightarrow 6.5)

^a Withdrawn after the second vaccine (cases 2, 4, and 6).

^b DTH size (mm).

^c ND, not done.

0.83×10^7 cells were obtained with 90% viability. By morphology, the harvested population of cells was $70\% \pm 15\%$ large dendritic-like cells and $20\% \pm 8\%$ 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. One patient who had anemia required blood transfusion before leukapheresis. There were no acute toxicities during or immediately after the i.v. DC infusion. No hematological, hepatic, pulmonary, or renal toxicities were observed in any patients, including three patients who were withdrawn after two vaccinations (Table 2).

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 CTL response for eight patients. Three patients were withdrawn after the second vaccination, and the fourth leukapheresis was not performed. One patient received all of the four immunizations; however, enough cell numbers of PBMCs could not be collected for CTL assay at the last leukapheresis. A CTL response was considered to be positive with a peptide-specific lytic activity exceeding 20% at an E:T ratio of

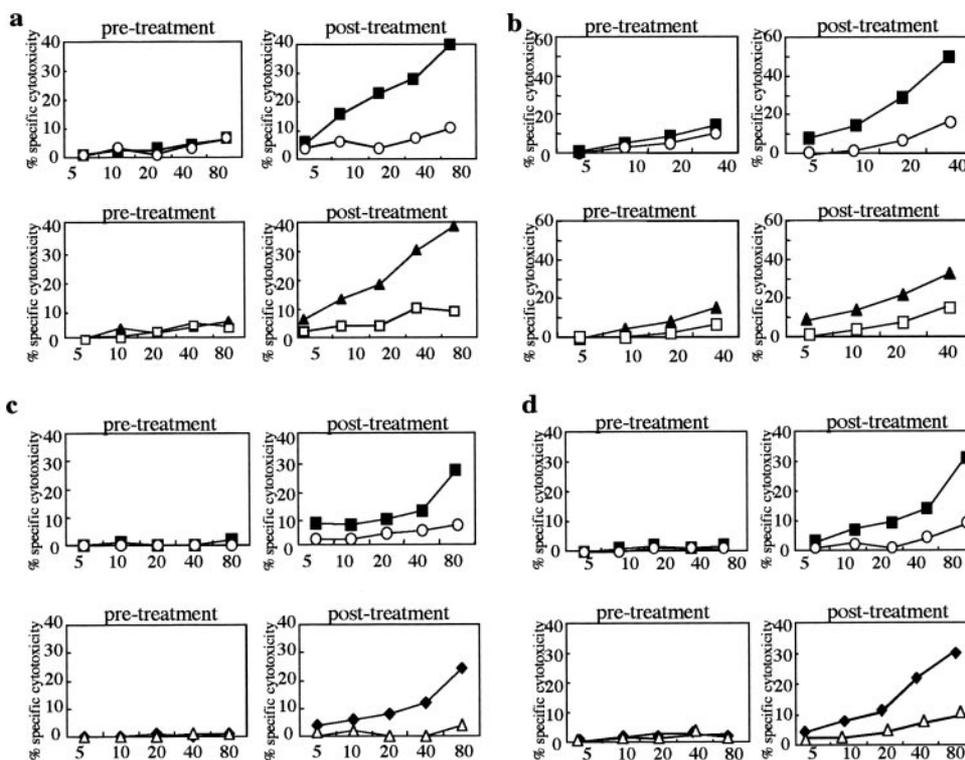


Fig. 1 The peptide-specific CTL responses were observed after DC vaccine treatment. The effector cells, as described in “Materials and Methods,” were obtained by stimulating PBMCs from the first leukapheresis and fourth leukapheresis. *a*, case 3; HLA-A2; and *b*, case 7; HLA-A2. Target cells for HLA-A2, 221(A2.1) [HLA-A2(+), MAGE-3(-)] were either pulsed (■) or not pulsed (○) with MAGE-3.A2 peptide (FLWGPRALV). The effector cells of posttreatment but not of pretreatment were able to lyse target cells pulsed with MAGE-3.A2 peptide. The effector cells of posttreatment were also able to lyse KATO-III cells [▲, MAGE-3(+), HLA-A2(+)] but not to lyse Raji cells [□, MAGE-3 (-), HLA-A2(-)]. *c*, case 5; HLA-A24; and *d*, case 9; HLA-A24. Target cells for HLA-A24, TISI [HLA-A24(+), MAGE-3(-)] were either pulsed (■) or not pulsed (○) with MAGE-3.A24 peptide (IMPKAGLLI). The effector cells of posttreatment but not of pretreatment were able to lyse target cells pulsed with MAGE-3.A24 peptide. The effector cells of posttreatment were also able to lyse WiDr cells [◆, MAGE-3(+), HLA-A24(+)] but not to lyse KATO-III cells [△, MAGE-3(+), HLA-A24(-)].

80:1 or 40:1 (19). 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 (7). Before vaccination, CTL precursor frequencies were low or undetectable. On the other hand, the peptide-specific CTL responses were observed in four of eight patients after vaccination (Fig. 1).

Peptide-specific DTH reactions were not observed before vaccination in any patient; however, there were three patients in which we observed DTH reactions after the fourth vaccination (Table 2). Intracellular cytokine analysis was performed for six patients. In three of six patients, the ratio of IFN-γ:IL-4 of CD4-positive cells increased after vaccination compared with before vaccination (Fig. 2).

Clinical Response. Table 3 summarizes the clinical response for the 12 patients individually. In seven 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 three patients (cases 8, 9, and 10). In case 8, perigastric metastatic lymph nodes (right cardia) that were resistant to prior chemotherapy and irradiation regressed, and middle and lower esophagectomy was performed after the DC vaccine protocol. Pathological and immunohistochemical

evaluation was done for resected specimens. Esophageal squamous cell carcinoma tissue showed necrosis and fibrosis, and T lymphocytes (mainly CD8-positive) infiltrated around some viable cancer cells nests that expressed MAGE-3 protein, but not within cancer nests (Fig. 3). On the other hand, few lymphocytes were observed infiltrating around cancer cells, which expressed no MAGE-3 protein. In case 9, metastatic lesions of right supraclavicular lymph nodes regressed after the second vaccine, and also the hoarseness was improved. In case 10, atelectasis of the left lung was improved because of tumor regression of a left huge metastatic thoracic tumor (Fig. 4). Because of the tumor regression, dyspnea became better. Four patients showed an improvement in performance status, and four patients maintained the performance status compared with their status pre-treatment.

DISCUSSION

Gastrointestinal carcinomas, such as esophageal carcinoma, gastric carcinoma, and colorectal carcinoma may be curable by surgery, but the cure rate is moderate to poor dependent on the extent of disease. Chemotherapy may have some

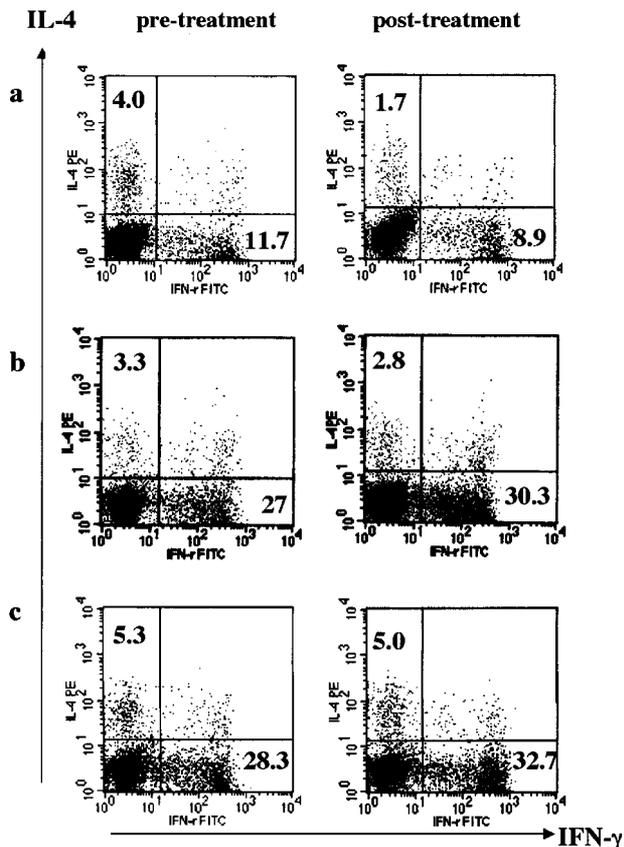


Fig. 2 Flow cytometric analysis of IFN- γ and IL-4 in the cytoplasm of peripheral CD4-positive T cells as described in "Materials and Methods." The ratio of IFN- γ :IL-4 of CD4-positive cells (Th1:Th2) increased in number after vaccination in three patients [a, case 8 pretreatment, 11.7:4.0 (2.9); posttreatment, 8.9:1.7 (5.3); b, case 10 pretreatment, 27:3.3 (8.2); posttreatment, 30.3:2.8 (10.8); and c, case 12 pretreatment, 28.3:5.3 (5.3); posttreatment, 32.7:5.0 (6.5)].

benefit; however, there is no standard regimen for advanced gastrointestinal carcinomas, and adverse events sometimes occur. Cellular immunotherapy for gastrointestinal carcinomas is considered as an alternative treatment approach. Active immunotherapy using autologous tumor cells and BCG showed some effects for stage II colon cancer as an adjuvant therapy (22). Adoptive transfer of lymphokine-activated killer cells with IL-2 showed objective responses in some patients with colorectal cancer (23). However, for these types of treatment, it is difficult to evaluate the immune response to cancer; therefore, these therapeutic effects were not fully understood.

The cancer testis antigens such as MAGE are the most promising candidates for tumor-specific immunotherapy of cancer, because specific therapeutic approaches require selectively expressed antigens. *MAGE* genes are not expressed in normal tissues except testis, and these cells do not express MHC class I molecules, and they do not present MAGE peptides at their surface. Therefore, immunotherapy with MAGE-derived antigens could avoid unexpected diseases such as autoimmune disease. No significant toxicity was observed in clinical trials with MAGE-3 peptide for melanoma patients (10, 13). Our

Table 3 Response to DC vaccine therapy (II)

Case ^a	Tumor marker ^b	Clinical response ^c	PS score ^d
1	Decrease CA 19-9; 65 \rightarrow 14	PD	2 \rightarrow 1
2	Increase CA 19-9; 2023 \rightarrow 9883	PD	2 \rightarrow 4
3	Decrease CEA; 3130 \rightarrow 2102	PD	2 \rightarrow 3
4	Decrease CEA; 161 \rightarrow 115	PD	2 \rightarrow 4
5	Decrease CEA; 274 \rightarrow 214	PD	1 \rightarrow 1
6	Increase CEA; 2903 \rightarrow 7704	PD	2 \rightarrow 4
7	Increase SCC; 1.8 \rightarrow 3.5	PD	1 \rightarrow 1
8	Decrease SCC; 2.0 \rightarrow 0.8	MR (3.0 \times 2.5 \rightarrow 2.0 \times 2.0)	2 \rightarrow 1
9	Decrease SCC; 1.7 \rightarrow 1.0	MR (6.5 \times 4.0 \rightarrow 5.5 \times 3.5)	2 \rightarrow 1
10	Decrease CEA; 29 \rightarrow 15	MR (14 \times 12 \rightarrow 11 \times 9.0)	2 \rightarrow 1
11	Increase CA 19-9; 42 \rightarrow 55	PD	1 \rightarrow 1
12	Increase CEA; 12 \rightarrow 53	PD	1 \rightarrow 1

^a Withdrawn after the second vaccine (cases 2, 4, and 6).

^b Tumor marker, CEA (ng/ml), CA 19-9 (units/ml), or SCC (ng/ml).

^c MR, tumor size (cm).

^d ECOG performance status score.

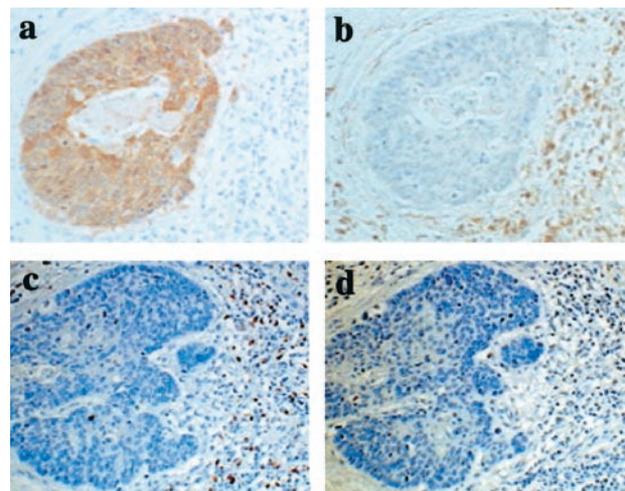
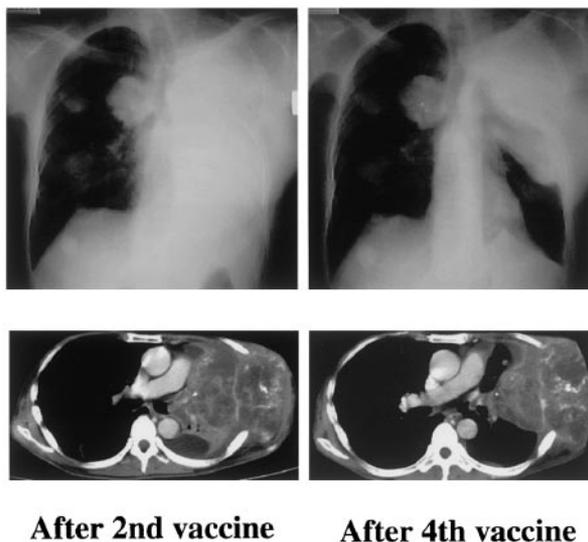


Fig. 3 Immunohistochemical analysis of the resected esophageal squamous cell carcinoma tissue after vaccination protocol in case 8. Some of the viable cancer nests expressed MAGE-3 protein (a, immunoperoxidase staining with 57B). Marked T lymphocytes (b, immunoperoxidase staining with UCHL-1) infiltrated surrounding tumor nests, which are composed of mainly CD8-positive lymphocytes (c, immunoperoxidase staining with C8/144B) and few CD4-positive lymphocytes (d, immunoperoxidase staining with 1F6; original magnification \times 100).

clinical trial also found no significant toxicity; therefore, MAGE peptide is considered an ideal target for gastrointestinal carcinomas.

There are some published clinical studies (12, 13, 24, 25)



After 2nd vaccine **After 4th vaccine**

Fig. 4 Clinical response to DC vaccination. Chest X-ray and computed tomography scans of case 10 showed tumor regression of the left chest wall tumor and improvement of atelectasis of the left lung after the fourth vaccine.

of DC vaccination with HLA-restricted peptides for cancer immunotherapy. From these pilot trials, DC vaccinations have established the general safety and feasibility of this type of approach, in addition to demonstrating immunological and clinical response for several tumor types. From our clinical trial, this type of treatment is also applicable for gastrointestinal carcinomas. Instead of peptides, the use of the whole protein (26), RNA (27), tumor lysate (12), or hybrid cells (28) could be presented to T lymphocytes as tumor antigens by DCs. In future studies, we should clarify which type of antigen induces the most effective immune response to cancer *in vivo*. Furthermore, the route of administration is also an important factor to elicit immune response. Because safe and effective immunization with antigen-pulsed DCs injected *i.v.* has been demonstrated in clinical studies (25, 26), we injected the DCs *i.v.* after pulsing them with peptides in this study. However, other routes of DCs (such as intradermal, *s.c.*, or intratumoral) have also been reported in some clinical trials (12, 13). Additional studies would be needed to decide the most appropriate route for immunization. Morse *et al.* (29) evaluated the pattern of distribution of DCs labeled with indium-111 oxyquinoline after *i.v.*, *s.c.*, and intradermal injection. The DC injected *i.v.* localized in the lungs and then redistributed to the liver, spleen, and bone marrow (reticuloendothelial system). On the other hand, DC injected intradermally migrated to the regional lymph nodes. We should clarify which site of T-cell contact will lead to a greater antigen-specific immune response for these diseases in another clinical protocol.

In the present study, peptide-specific immune responses were recognized in some patients by *in vitro* CTL precursor assay (four of eight) and DTH response (three of eight). However, there is no direct evidence of a correlation between the immune response and clinical tumor regression. Marchand *et al.* (10) also reported that MAGE-3.A1 peptide vaccination showed

no evidence for a CTL response in the blood of the four patients who were analyzed, including two who displayed complete tumor regression. Rosenberg *et al.* (30) reported that IL-2 administration in addition to modified gp 100 peptide, which showed clinical tumor regression in many patients, reduced the ability to detect antipeptide precursors in the peripheral circulation, compared with peptide alone, which showed no clinical response. This discrepancy between the development of antitumor precursors and clinical response is not clearly understood. Another new reliable method is required to detect immune responses against antigenic peptides. HLA tetramer may become a more sensitive and promising monitoring method to evaluate CTL precursors (31). We examined the local immune response in the resected specimens of patient 10. The specific immune response for tumor cells, which were expressing MAGE-3, may occur after vaccination. CD8-positive T lymphocytes were infiltrated around the tumor nests but not within tumor-expressing MAGE-3 protein in resected esophageal carcinoma tissue. On the other hand, there were few infiltrating T cells around the tumor nests expressing no MAGE-3 protein. Naito *et al.* (32) reported that T-cell infiltrates within cancer cell nests were associated with a favorable outcome; therefore, clinical response with tumor regression might be only a minor response in this case.

Two types of Th are categorized as Th1 and Th2 on the basis of their cytokine production. Th1 cells produce mainly IFN- γ and mediate cellular immune response, whereas Th2 cells produce mainly IL-4 and mediate humoral responses (33). A Th2-subset dominance among peripheral blood T lymphocytes was shown in patients with gastrointestinal carcinoma (34). In the present study, we observed the ratio of IFN- γ /IL-4 of CD4-positive cells (Th1/Th2) increased after vaccination in two 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.

In the future, consideration should be given to this type of immunotherapy with other treatment strategies to achieve a greater vaccine effect. The concurrent systemic administration of cytokines such as granulocyte macrophage colony-stimulating factor (35) or IL-2 (36) may enhance the efficacy of tumor vaccines. Using mature DCs may induce more effective immune response (13). In addition, vaccine therapy may be considered to be best suited either for patients at an early stage of disease or after surgical reduction of the tumor burden (11). 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.

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