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ORIGINAL ARTICLE

**Tsuyoshi Itoh · Yuji Ueda · Ichiro Kawashima
Ikuei Nukaya · Hitoshi Fujiwara · Nobuaki Fuji
Tetsuro Yamashita · Tetsunori Yoshimura
Kaori Okugawa · Tomoko Iwasaki · Mitsuko Ideno
Kazutoh Takesako · Masakazu Mitsuhashi
Kunzo Orita · Hisakazu Yamagishi**

Immunotherapy of solid cancer using dendritic cells pulsed with the HLA-A24-restricted peptide of carcinoembryonic antigen

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Abstract Carcinoembryonic antigen (CEA), an oncofetal glycoprotein overexpressed in most gastrointestinal and lung cancers, is a candidate molecule for cancer immunotherapy. Recently, a CEA-derived 9-mer peptide, CEA652 (TYACFVSNL), has been identified as the epitope of cytotoxic T lymphocytes restricted with human leukocyte antigen (HLA)-A24, which is present in 60% of the Japanese population and in some Caucasians. The authors performed a clinical study of a vaccine using autologous dendritic cells (DCs) pulsed with CEA652 and adjuvant cytokines, natural human interferon alpha (nhuIFN-α), and natural human tumor necrosis factor alpha (nhuTNF-α), for the treatment of patients with CEA-expressing advanced metastatic malignancies. Ten HLA-A24 patients with advanced digestive tract or lung cancer were enrolled in the study to assess toxicity, tolerability and immune responses to the vaccine. DCs were generated from plastic adherent monocytes of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (PBMCs) in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4). Generated DCs showing an immature phenotype were loaded with CEA652 and injected into patients intradermally and subcutaneously with 50% of the dose administered by each route every 2 weeks for a total of ten vaccinations. The total dose of administered DCs ranged from 2.7×10^7 cells to 1.6×10^8 cells. Adjuvant cytokines, i.e., 1×10^6 U/body of nhuIFN-α and nhuTNF-α, were administered to patients twice a week during the vaccination period. No severe toxicity directly attributable to the treatment was observed, and the vaccine was well tolerated. In the delayed-type hypersensitivity (DTH) skin test, two patients showed a positive skin response to peptide-pulsed DCs after vaccination, although none of the patients tested positive prior to vaccination. In the two patients who showed a positive skin response disease remained stable for 6 and 9 months respectively. These results suggest that active immunization using DCs pulsed with CEA652 peptide in combination with the administration of adjuvant cytokines is a safe and feasible treatment procedure.

Keywords Cancer vaccine · Cytokine · DTH · Gastrointestinal cancer · Lung cancer

T. Itoh (✉) · Y. Ueda · H. Fujiwara · N. Fuji · T. Yamashita
T. Yoshimura · K. Okugawa · H. Yamagishi
Department of Digestive Surgery,
Kyoto Prefectural University of Medicine,
465 Kajii-cho, Kamigyo-ku,
Kyoto 602-0841, Japan
E-mail: tito@koto.kpu-m.ac.jp
Tel.: +81-75-2515527
Fax: +81-75-2515522

I. Kawashima · I. Nukaya · T. Iwasaki · M. Ideno · K. Takesako
Biotechnology Research Laboratories,
Takara Shuzo Co. Ltd., Otsu,
Shiga 520-2193, Japan

M. Mitsuhashi · K. Orita
Hayashibara Biochemical Laboratories Inc.,
2-3 Shimoishii 1-chome, Okayama 700-0907,
Japan

Introduction

There are several strategies for eliciting an antigen-specific response using the cytotoxic T lymphocyte (CTL) epitope peptide in cancer immunotherapy. Among these strategies, dendritic cell (DC)-based active immunotherapy is one of the most promising approaches for the treatment of cancer [23]. DCs are professional antigen-presenting cells that can induce a strong cellular immune response. There is an abundant expression of co-stimulatory molecules on their surface, and they migrate to lymphoid organs where they activate antigen-specific T-cells. They have been shown to stimulate both memory and naive T-cell responses in vitro [2]. Several studies in which immunization with antigen-pulsed DCs efficiently elicited CTL responses in melanoma patients have been

published [17, 18, 27], but few reports have been made on other malignancies such as gastrointestinal and lung cancer. We therefore conducted a clinical study on active immunotherapy for patients with carcinoembryonic antigen (CEA)-expressing metastases originating from gastrointestinal and lung cancer. In the current study, DCs pulsed with a peptide derived from CEA were administered to patients in combination with two cytokines, interferon alpha (IFN-α) and tumor necrosis factor alpha (TNF-α), in an adjuvant setting.

Although CEA is an 18-kDa glycoprotein found in normal adult colonic mucosa and fetal tissues, it is widely used as a serological marker of gastrointestinal cancer because of its overexpression in the latter [26]. It is also an adhesion molecule and may be involved in metastasis by mediating the attachment of tumor cells to normal cells [3]. Thus, CEA-targeting immunotherapy might prevent the development of metastases. CEA can be recognized as a self-antigen by the immune system because of its presence in normal tissues, and thus most individuals including cancer patients should be immunologically tolerant to this antigen. However, a CTL response against CEA was elicited by recombinant vaccinia–CEA vaccine in a phase I clinical study and a human leukocyte antigen (HLA)-A2.1-restricted CTL epitope peptide of CEA, CAP-1, was identified [8, 29]. Furthermore, a nonamer peptide obtained from healthy humans, CEA652 (TYACFVSNL), was shown to induce HLA-A24-restricted CTL in vitro [19]. These findings suggest that not only healthy individuals but also cancer patients are non-tolerant to CEA. We therefore selected CEA as the antigen of choice for active immunotherapy and used CEA652, restricted with HLA-A24, the most frequent allele in Japan, for loading onto DC.

The use of cytokines as adjuvants might play an important role in the further development of cancer vaccine therapy. We used IFN-α and TNF-α, which have a variety of functions affecting antigen-presenting cells (APC) and tumor cells, including the following: (1) upregulation of the expression of major histocompatibility complex (MHC)-class I molecules in tumor cells [1, 9, 10, 24, 30]; (2) upregulation of tumor-associated antigens (TAAs) [1, 10] including CEA [30] in tumor cell lines; and (3) promotion of DC maturation in the developmental stages [4, 13]. We therefore considered that they might be effective not only in the immunizing phase but also in the effector phase in DC-based cancer immunotherapy. A clinical trial on the systemic administration of IFN-α and TNF-α to advanced cancer patients performed by Orita et al. showed an antitumor response in 14 out of 112 patients (12.5%) [20]. However, at the dose used in the current study, no clinical response was elicited in their trial.

We selected HLA-A24 as the target allele because it is the most frequently occurring allele in the Japanese population, much more frequent than HLA-A2.1 [5], and often present in other Asian populations and also in Caucasians. We evaluated safety mainly by monitoring acute and chronic toxicity including CEA-specific autoimmune adverse reactions. We also studied the feasibility of using DC vaccine combined with adjuvant cytokines in comparison with administering DC vaccine alone.

Materials and methods

Patients

Ten patients were referred from the gastrointestinal surgery clinic of Kyoto Prefectural University of Medicine from December 1998 to April 1999. Eligible patients had advanced digestive tract or lung cancer that was refractory to conventional chemotherapy or radiotherapy. Entry criteria were as follows: (1) serological confirmation of HLA-A24; (2) metastatic cancer expressing CEA as defined by immunohistochemical analysis; (3) a performance status (Eastern Cooperative Oncology Group) of 0 or 1. Exclusion criteria were as follows: (1) chemotherapy or radiotherapy 4 weeks prior to treatment; (2) presence of acute or chronic viral infection including HIV, hepatitis B or C; (3) organic dysfunction of the liver, kidneys or dysfunction of hematological origin; or (4) history of autoimmune disease.

Study design

All patients were required to give informed consent prior to participation in the study (the form was approved by the Institutional Ethics Review Committee, Kyoto Prefectural University of Medicine). Peripheral blood was subjected to leukapheresis using a Blood Cell Separator CS-3000 (Baxter, Deerfield, Ill.) to obtain peripheral blood mononuclear cells (PBMCs) after mobilization by recombinant human granulocyte colony-stimulating factor (rhG-CSF) [Gran (filgrastim); Kirin Brewery, Gunma, Japan] administered at a dose of 10 μg/kg once daily for 5 consecutive days. The leukapheresis products were used as the source of DC progenitors. Patients received vaccination on an outpatient basis, and were monitored for acute toxicity for at least 3 h post-administration in the hospital. DC vaccine preparation (0.5 ml/dose) was injected intradermally and subcutaneously at the same site in the inguinal region using a tuberculin syringe with a 27-G needle. Treatment consisted of ten vaccinations at 2-week intervals. The first three of the ten patients enrolled received DC vaccine alone, and the other seven patients received DC vaccine, followed by the administration of adjuvant cytokines: natural human interferon-alpha (nhuIFN-α) and natural human tumor necrosis factor-alpha (nhuTNF-α). In the latter group, DC vaccine preparation was injected at day 0, followed by intravenous injections of the adjuvant cytokines nhuIFN-α and nhuTNF-α (both at 1×10^6 U; kindly provided by Hayashibara Biochemical Laboratories, Japan) twice a week at days 1 and 4. To monitor delayed-type hypersensitivity (DTH) response, skin tests were performed at all therapeutic intervals. A positive skin-test reaction was defined as erythema and skin induration >5 mm in diameter after 48 h post-injection. DCs (1×10^5 cells) pulsed with CEA652 at a concentration of 40 μg/ml were injected on alternate sides of the inguinal region. As a control, DCs without peptide were tested simultaneously. Patients attended hospital 2 days after vaccination, and the diameter of the local skin induration caused by the DTH skin test was measured. During the vaccination period, blood samples were drawn from patients to monitor serum CEA levels every 2 weeks. Peripheral blood leukocytes and granulocytes were counted, and gastrointestinal tract clinical symptoms were carefully monitored. Adverse reactions were evaluated in accordance with the criteria of the Japanese Society for Cancer Therapy, based on the criteria of the World Health Organization (WHO). X-rays, computed tomography (CT) and magnetic resonance imaging (MRI) were conducted at 4- to 8-week intervals and used to evaluate the development of metastatic lesions. For further analysis, patients underwent leukapheresis with an exchange of approximately 5,000 ml of blood within 1 week before the onset of G-CSF-mobilization and about 2 weeks after

the final vaccination to collect PBMCs, which were frozen and stored in liquid nitrogen until use.

Generation of DCs and vaccine preparation

The leukapheresis products were separated by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Sweden). PBMCs obtained were suspended in CP-1 (Kyokuto Seiyaku, Tokyo, Japan) mixed with an equal volume of RPMI 1640 (Nikken, Kyoto, Japan) at about 5×10^7 cells/ml, and divided into vials each containing 2 ml of the cell suspension. All vials were stored in a liquid nitrogen tank. Two vials were used for the preparation of DC vaccine for each treatment. After thawing, PBMCs were suspended in 20 ml of RPMI 1640 supplemented with 5% heat-inactivated pooled human plasma (Japanese Red Cross Society) and gentamycin (complete medium; Fujisawa Pharmaceutical, Osaka, Japan), and plated in 75-cm^2 cell culture flasks. The flasks were incubated in a 5% CO_2 incubator at 37°C for 2 h, and non-adherent cells were removed. Adherent cells were cultured in 30 ml of complete medium containing 1,000 U/ml each of recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF; Kirin) and recombinant human interleukin-4 (rhIL-4; Genzyme, Minneapolis, Minn.). After culturing for 7 days, DCs were harvested, washed twice, and suspended in 1 ml of phosphate-buffered saline (PBS) containing 1% human albumin (Fujisawa). CEA652 peptide, TYACFVSNL (>95% purity and endotoxin-free; Takara, Shiga, Japan) was dissolved at 40 μg/ml and added to the DC suspension. After 2 h, the cells were washed and prepared as a cell suspension in 1 ml of saline containing 1% human albumin, and used as the vaccine. The study protocol and the use of the peptide and cytokines were approved by the Institutional Ethics Review Committee, Kyoto Prefectural University of Medicine.

Phenotypic characterization of DCs was carried out using FACSCalibur (Becton Dickinson, San Jose, Calif.) and CellQuest software. Immunofluorescent staining was performed using the following monoclonal antibodies (mAbs): W6/32 for HLA class I; CR3/43 for HLA-DP/DQ/DR; FITC-conjugated anti-CD3 (Dako A/S, Denmark); anti-CD14; anti-CD19; anti-CD34; PE-conjugated anti-CD80 (Becton Dickinson); anti-CD83 (Immunotech, France); and anti-CD86 (Ancell, Bayport, Minn.). Cell viability determined by the trypan blue exclusion test was >95%. DC preparations were confirmed to be endotoxin-free (<10 pg/ml in the supernatant).

Assessment of Ag recognition by CTL

To determine the immune response to CEA652 peptide in vitro, we performed a semi-quantitative assessment of CTL generated from PBMCs obtained pre- and post-vaccination by standard ^{51}Cr-release assay. CTL induction was carried out using DCs as described previously [28]. Briefly, DCs were generated from monocytes as described above, and pulsed with CEA652 for 4 h in PBS containing 1% bovine serum albumin (BSA). The peptide-pulsed DCs were then irradiated and mixed with $CD8^+$ T cells obtained by positive selection from PBMCs pre- and post-vaccination. The cultures were carried out in 48-well plates. On days 7 and 14, the T-cell cultures were restimulated with autologous peptide-pulsed adherent monocytes. After four rounds of restimulation, each well was tested separately on day 36 for cytotoxic activity by 6-h ^{51}Cr-release assay. Target cells were peptide-pulsed TISI cells, an Epstein-Barr virus (EBV)-transformed HLA-A24-positive B cell line, and TISI cells without peptide. All of the assays were performed in the presence of a 30-fold excess of unlabeled K562 to block non-specific natural killer (NK) activity. Wells were scored positive if the cytotoxicity of peptide-pulsed TISI targets was 12% above that of targets without peptide.

Results

Patient characteristics

The characteristics of the ten patients enrolled in this study have been listed in Table 1. The study group consisted of eight women and two men aged from 28 to 69 years, with a median age of 55 years. Primary tumors were located in the colon or rectum in seven patients, the lungs in two patients and the stomach in one patient. CEA expression was confirmed in specimens obtained at resection or biopsy. Multiple sites of involvement were detected in the liver of four patients and the lungs of two patients. Three patients had peritoneal metastases, which are most frequently observed in cases of gastrointestinal cancers and are refractory to conventional therapy. All patients had increased serum CEA levels, ranging from 4 to 394 ng/ml (cut-off value: 2.5 ng/ml) and had previously undergone surgical resection of the primary lesions. Unresected metastatic lesions, which were either already present at the time of surgery or became apparent after surgery, were treated with conventional chemotherapy (5-fluorouracil in five cases, and epidoxorubicin and leucobolin in the other cases) or by radiotherapy prior to inclusion in this study. An average period of 7 weks (range: 4–16 weeks) had elapsed between their last treatment and inclusion in this study. The mean blood leukocyte count was $4.6\pm1.2\times10^3/\mu l$, i.e., $24.4\pm10.8\%$ lymphocytes, $8.31\pm2.5\%$ monocytes and $67.8\pm12.8\%$ granulocytes. The performance status of all patients was scored as 0.

Pre-vaccination immunological status was examined using PBMCs obtained before therapy. The lymphocyte

Table 1 Patient characteristics (*S* surgery, *C* chemotherapy, *R* radiotherapy)

Patient no.	Age (years)/gender	HLA-A allele	Primary lesion	Metastatic site	Prior therapy[a]
1	54/F	24	Rectal	Lung, pelvis	S, C
2	64/F	11/24	Colon	Liver, lymph nodes	S, C
3	61/F	24	Colon	Liver, peritoneum	S, C
4	28/F	2/24	Colon	Peritoneum	S, C
5	33/F	2/24	Stomach	Peritoneum	S, C
6	69/M	2/24	Lung	Pleura, lymph nodes	S, C
7	57/F	24/26	Rectal	Lung, adrenal	S, C
8	56/M	2/24	Rectal	Liver	S, C
9	52/F	24/33	Lung	Bone	S, C, R
10	42/F	24	Colon	Liver	S, C

fraction of PBMCs was 60.8 ± 9.8%, and the CD8/4 ratio was 0.7 ± 0.4. CEA652 peptide-specific CTL was induced in four patients in vitro before therapy. Two patients with much lower CD8/4 ratios (no. 3: 0.17, no. 7: 0.2) than healthy subjects (range: 0.67–1.0) tested negative for the induction of peptide-specific CTL. However, there were no significant correlations between the peptide-specific CTL response and immunological parameters, including the CD8/4 ratio and phytohemagglutinin (PHA)-stimulated proliferation of lymphocytes (data not shown).

Characteristics of DCs generated from G-CSF-mobilized PBMCs

The results of leukapheresis and the characteristics of the generated DCs are shown in Table 2. We obtained $2.5 \pm 1.4 \times 10^6$ cells/ml as PBMCs with an exchange of approximately 6,000 ml of blood volume by leukapheresis after G-CSF mobilization. The G-CSF-mobilized PBMCs contained $< 1.0\%$ $CD34^+$ hematopoietic stem cells in most cases. The pre- and post-mobilization lymphocyte/monocyte ratios were 2.86 and 1.18 respectively, indicating that the mobilized PBMCs were rich in monocytes. DCs were generated from the adherent fraction of mobilized PBMCs with a yield of 8.6 ± 8.1% of PBMCs in the steps before cell adherence. By FACS analysis, the harvested population of cells consisted of 60% large DCs with high levels of expression of HLA-ABC, HLA-DP/DQ/DR and CD86; no expression of CD14; and low levels of expression of CD80 and CD83 (Fig. 1); and 40% small cells mainly expressing CD3 (data not shown). These results indicated that cells with typical DC morphology and surface phenotypes were successfully generated in all patients with advanced cancer.

Patient treatment and adverse effects

Table 3 summarizes the results of treatments, i.e., the toxicities and immunological and clinical responses. Results concerning the first three patients who received DC vaccine alone showed no immunological (DTH) or clinical responses. All seven patients who were then treated by DC vaccine combined with cytokines IFN-α and TNF-α were evaluated for toxicity and clinical immunological response after ten vaccinations. No major acute toxicities (grade III or IV), anaphylactic reactions, or changes in vital signs were observed in any case. Local induration at the vaccination site occurred in two patients (Nos. 7 and 9) after the third vaccination, which by the fifth vaccination had deteriorated to 60 and 55 mm, respectively. Other adverse effects were recorded in two other patients: one patient (no. 1) showed an increase in liver function test results and leukocytosis, both of which were probably caused by biliary tract infection due to the tumor; anemia due to disease progression was observed in the other patient (no. 2). The adverse effects observed in patients nos. 1 and 2 were attributed mainly to rapid tumor growth rather than to the effect of the vaccine. Since toxicities of the vaccine were mild and not dose-related, it was concluded that it was well tolerated. Diarrhea or other autoimmune disease-related symptoms of the gastrointestinal tract were not observed in any of the patients. Moreover, there were no significant differences in the white blood cell counts pre- and post-vaccination (paired *t*-test). The peripheral blood after vaccination contained 25.2 ± 14.5% lymphocytes and 8.4 ± 3.5% monocytes, showing no significant change pre- and post-vaccination (paired *t*-test). Granulocyte counts amounted to 67.8 ± 12.8% and 66.3 ± 15.8% before and after vaccination respectively, indicating that no patient had granulocytopenia caused by an autoimmune response.

As shown in Table 3, a continuous decrease of serum CEA levels was observed in one patient (no. 9): a marked decrease, i.e., from 394 to 49 was observed over the treatment period. CT examination showed tumor involvement of the sacral bone, which remained stable for 6 months without the appearance of new lesions or further development of existing lesions. Later, however, the disease gradually progressed without any increase in CEA levels thereafter, and the patient died 14 months after the start of treatment. The rise of serum CEA levels was controlled in the two other patients (nos. 6 and 7). In patient no. 7, who had multiple lung and adrenal metastases of rectal origin disease remained stable for 9 months, and she is still alive 19 months after treatment. However, the other patient (no. 6) has had progressive disease.

Table 2 DC generation from PBMCs mobilized with G-CSF

Leukapheresis		
Blood volume processed	PBMCs harvested	
6014 ± 1510 ml	$2.5 \pm 1.4 \times 10^6$ cells/ml	
DC generation		
PBMCs prepared	$1.5 \pm 1.4 \times 10^8$ cells	
DCs harvested	$1.0 \pm 0.7 \times 10^7$ cells	
% DC yield	8.6 ± 8.1%	
DC surface phenotype	Class I	99.7 ± 5%
	Class II	99.9 ± 8%
	CD14	6.8 ± 14%
	CD80	15.6 ± 9%
	CD83	7.8 ± 5%
	CD86	98.6 ± 11%

Post-vaccination immunological response

DTH responses which were evaluated at each vaccination in all patients were positive, with skin induration and erythema in reaction to the peptide-pulsed DCs in two patients (nos. 7 and 9; Table 3). Although faint erythema was observed ranging from 1 to 3 mm in diameter after the third vaccination, strong reactions with skin induration of > 25 mm were noted in patients nos.

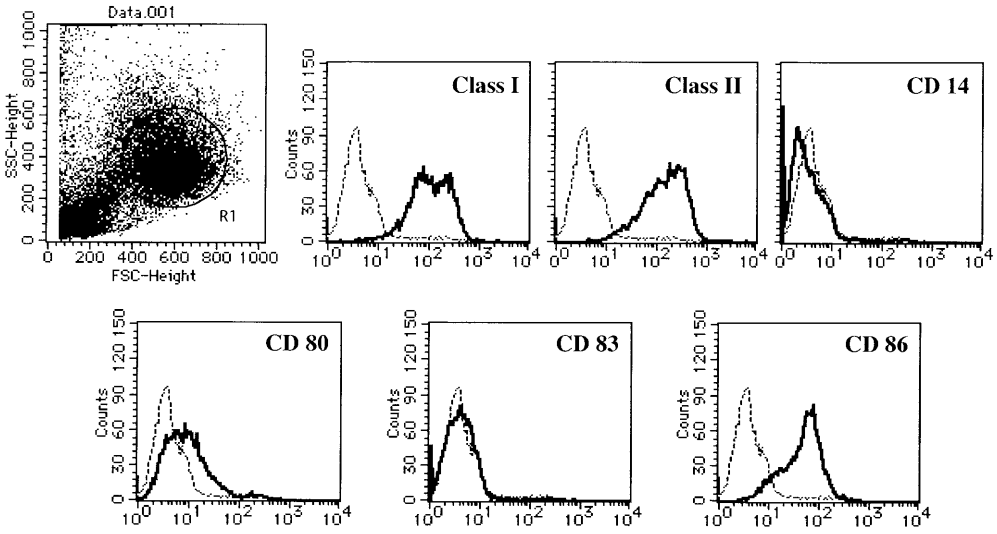

Fig. 1 Cell-surface membrane phenotype of generated DCs which were administered to patients. In this representative analysis, the cells expressed high levels of HLA class I, class II and CD86, in contrast to low levels of CD14 and CD83, a marker for mature DC, consistent with prior reports on in vitro-cultured DCs

7 and 9 after the eighth and the seventh vaccinations, respectively. The skin test against purified protein derivative of tuberculin (PPD) as a positive control of recall response was weakly positive in patients nos. 7 and 9 (8 and 11 mm, respectively). Their CD8/4 ratios were >1.0 (no. 7: 1.02; no. 9: 1.18), and total cell counts of administered DCs until the time when a positive DTH response was observed were 7.5×10^7 and 7.9×10^7, respectively.

To evaluate the immunological response in vitro, CEA652 peptide-specific CTL induction was performed in these two patients using leukapheresis products obtained after all vaccinations. Although we also used the ELISPOT assay, antigen-specific IFN-γ-producing cells were detected only after PBMC boosting by peptide stimulation. It did not appear to be suited for assessment in the present study due to the non-specific reactions detected (data not shown). We therefore performed a semi-quantitative assay by comparing the efficiency of CTL induction at pre- and post-immunization for assessment of the immune response in vitro. As shown in Fig. 2a, enhancement of the peptide-specific CTL response after vaccination was observed in patient no. 7: before vaccination no wells showed a peptide-specific CTL positive response in the 48 wells tested, but after vaccination five out of the 40 wells (12.5%) scored positive with high peptide-specific cytotoxicity. Boosted CTLs in vitro showed high IFN-γ but not IL-10 production in a peptide-specific fashion (Fig. 2b). However, no enhancement of CTL peptide-specific response was observed in patient no. 9 (before: 3/32 wells; after: 1/24 wells) or in the other patients evaluated.

Discussion

Antigen-specific active immunotherapy may induce an autoimmune response to self-antigens expressed in normal tissues [12]. Thus, autoimmune adverse effects should be carefully assessed for the further development of CEA-targeting immunotherapy. A major aim of this study was to examine the safety and feasibility of using a vaccine consisting of DCs pulsed with CEA652 peptide and the adjuvant cytokines, IFN-α and TNF-α. CEA is expressed not only in gastrointestinal and lung epithelial tumor cells, but also weakly in normal colonic mucosa, and has a high homology with the CEA gene family such as biliary glycoprotein (BGP) and non-specific cross-reacting antigen (NCA) found in some normal tissues including granulocytes [25]. Its cross-reaction with BGP

Table 3 Patient treatment and toxicity (*PD* progressive disease, *SD* stable disease)

Patient	Treatment	No. of DCs administered	Toxicity	DTH	Clinical response	Serum CEA
1	DC alone	2.7×10^7	Grade1 (liver)	–	PD	↑
2	DC alone	5.3×10^7	Grade 1 (anemia)	–	PD	↑
3	DC alone	1.6×10^8	–	–	PD	↑
4	DC with cytokine	1.1×10^8	–	–	PD	↑
5	DC with cytokine	1.2×10^8	–	–	PD	↑
6	DC with cytokine	7.7×10^7	–	–	PD	→
7	DC with cytokine	8.5×10^7	Grade 1 (local)	+	SD	→
8	DC with cytokine	6.5×10^7	–	–	PD	↑
9	DC with cytokine	1.3×10^8	Grade 1 (local)	+	SD	↓
10	DC with cytokine	1.2×10^8	–	–	PD	↑

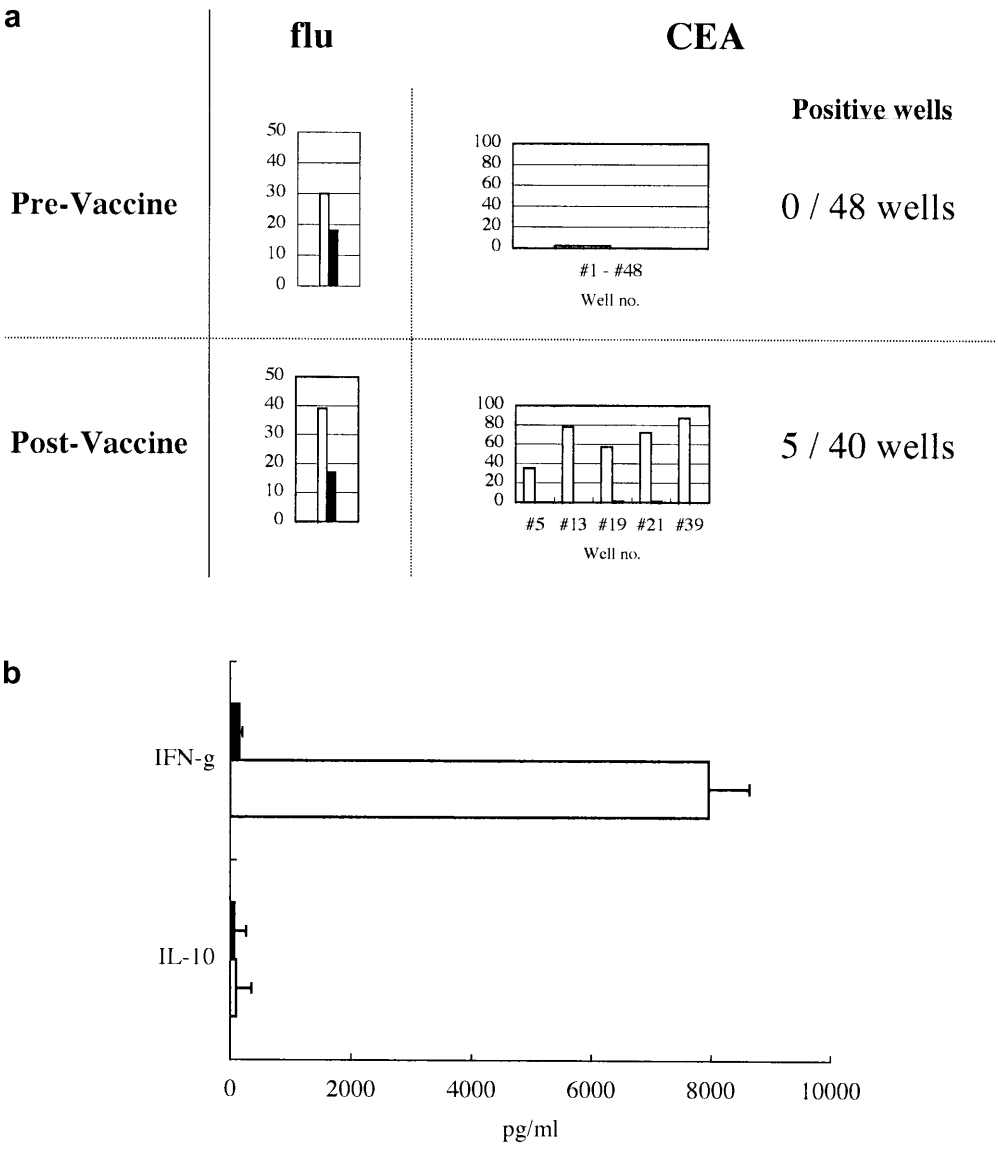

Fig. 2 **a** The peptide-specific CTL response was observed in patient no. 7 after treatment. Pre- and post-immunization $CD8^+$ T cells were stimulated with CEA652-pulsed autologous DCs (flu peptide (RFYIQMCTEL, A24-restricted peptide derived from influenza nucleoprotein)-pulsed DCs as controls). After four rounds of restimulation, each well was tested for cytotoxic activity by ^{51}Cr-release assay. Relevant peptide-pulsed (❑) or unpulsed (■) TISI cells were used as targets. One representative confirmed by repeated experiments. **b** IFN-γ production by boosted CTLs generated from post-immunization PBMCs. One$\times 10^5$ CTLs were co-cultured with peptide-pulsed (❑) or unpulsed (■) TISI cells in 96-well plates for 24 h, then culture supernatants were collected for determinations of IFN-γ and IL-10 by ELISA. The bars represent mean ± SD of triplicate samples

and NCA should be avoidable in this vaccine strategy, since the CEA652 peptide used in this study shows no homology with the counterpart of BGP or NCA (NCA50/90, NCA90), differing in more than three amino-acid residues [25]. None of our patients displayed any autoimmune reactions, i.e., adverse symptoms of the gastrointestinal tract or agranulocytosis. This finding was consistent with those of other clinical trials targeting CEA, including ALVAC-CEA [15], vaccinia-CEA [8] and CAP-1 peptide-pulsed DC [16], which also reported no autoimmune response. No major toxicities (grades 3 and 4) were observed in any of the patients, and the CEA652 vaccine was well tolerated.

In humans, DCs can be generated either from non-proliferating $CD14^+$ monocytes in the peripheral blood or from proliferating $CD34^+$ precursors. The former have been widely used for experimental and clinical purposes because of their accessibility. It is not, however, clear which source is suitable for clinical use in active immunotherapy. For preparation of DCs, we used monocytes mobilized with G-CSF, a method which was expected to make it clinically practical to generate DCs on a large scale. The leukapheresis products after G-CSF-mobilization were more abundant in monocytes, and consequently yielded 6-fold more DCs than without mobilization. The DCs generated from mobilized monocytes had a typical DC morphology, displaying a veiled and enlarged cell structure, and also exhibited high allo-stimulatory activity (data not shown). In addition, there was a high expression of class I, class II, and CD86, and no expression of CD14, which is typical for immature DCs. Although the use of G-CSF-mobilized PBMCs as a cell source for DC generation has not been fully evaluated, it has been suggested that G-CSF-mobilization could be useful for the large-scale preparation of DCs.

One endpoint of clinical studies on cancer vaccines is to generate an immune response to the antigen admin-

istered. At present, there is no gold standard for measuring the T-cell-mediated immune response elicited by the vaccines. For in vivo detection of the immune response, we performed a DTH skin test for the peptide antigen in all patients participating in the study. It is evident that the DTH response is a reflection of the development of systemic cell-mediated immunity and may be important for immunological monitoring after class I-restricted peptide immunization, as reported in some clinical studies [7, 16, 18]. In our study, no positive response was found in the first three patients who underwent DC vaccination only. However, the use of adjuvant cytokines elicited a positive response in two patients (nos. 7 and 9) out of seven after DC vaccination. These responses were not evident until after the seventh or the eighth injection. However, a local reaction at the vaccination site, which may be indicative of an immune response to the vaccine, was already observed after the third vaccination. Thus, it is suggested that the cell numbers used in the DTH skin test may be correlated with the magnitude of the response.

For further assessment of the immunological response, we performed an in vitro CTL induction experiment to examine the precursor frequency of peptide-specific CTL. The ELISPOT assay for detecting and enumerating single cytokine-secreting cells requires $> 1/10^5$ CTLs in peripheral blood lymphocytes [21]. Because the number of CTL precursors in our samples was estimated to be below the assay detection limit in the preliminary study, we therefore performed a semiquantitative assay by comparing the efficiency of CTL induction at pre- and post-immunization for assessment of the immune response in vitro. The experiment indicated that patient no. 7 had enhanced CTL precursors that recognized the CEA652 peptide (Fig. 2a), but, in contrast, patient no. 9 had no enhanced response, although both patients tested positive for the peptide in the DTH skin test. Our results could indicate that the DTH skin test is more sensitive for detecting the antigen-specific T-cell response, especially in cases with few CTL precursors for antigen peptide such as CEA652 peptide in the peripheral blood than the in vitro CTL induction experiment. Although the discrepancy between the negative response in enhancement of CTL and the positive DTH response observed in patient no. 9 remains to be addressed, the most intriguing finding was that the DTH response seen in patients nos. 7 and 9 was consistent with two out of the three cases in which stable or decreased serum CEA levels were identified. Discrepancies between the immunological response and the clinical response have been discussed previously [11, 14, 22]. Our observation that the DTH immune response in two patients was supported by a positive clinical response could therefore be significant. Thus, the DTH test might be useful as a surrogate marker for clinical effects.

It has recently been reported that the injection of immature DCs elicits antigen-specific inhibition of effector T cell function in humans [6]. The study showed that injection of immature DCs led to the appearance of peptide-specific IL-10-producing $CD8^+$ T cells with reduced IFN-γ production and which lacked NK activity. In our study, CTL generated from post-immunization T cells had high killer activity: 87% cytotoxicity at an E:T ratio of 10:1 (Fig. 2a). When post-immunization T cells were boosted in culture, high IFN-γ but not IL-10 production was detected upon relevant peptide stimulation in the supernatant by ELISA (Fig. 2b). These findings may indicate that mainly peptide-specific IFN-γ-producing T cells were predominantly expanded in patient no. 7 who received DC vaccination with adjuvant cytokines, and that cytokines played a role in vivo in the maturation of the administered DCs and in the prevention of specific inhibition of effector T cell function.

IFN-α and TNF-α were systemically administered at doses expected to induce two major adjuvant effects when combined with peptide-pulsed DCs: (1) to promote the maturation of infused DCs in vivo [4, 13]; and (2) to increase the number of MHC-peptide molecules for TAAs on tumor cells. The positive DTH response observed only in patients who received DC vaccination in combination with the administration of adjuvant cytokines may indicate that these cytokines can play a role in the augmentation of the immunological effects of the vaccine. However the enhanced CTL response detected in vitro was observed in only one patient, and thus further examination is warranted. On the other hand, stable or decreased serum CEA levels were identified in three out of seven patients who received adjuvant cytokines, whereas no response was apparent in patients not given the cytokines. Moreover, two out of the three patients with controlled serum CEA levels had stable disease for 6 and 9 months. These facts suggest that administration of IFN-α and TNF-α can enhance the clinical response. It is generally considered that cytokines that up-regulate the expression of MHC-class I molecules and TAAs should be suitable for DC vaccine therapy, since CTL recognize the complex molecules of peptide antigen and MHC-class I molecules presented on the cell surface. IFN-α and TNF-α up-regulate the expression of MHC-class I molecules and TAAs, including the CEA of tumor cell lines [1, 9, 10, 24, 30]. Therefore, it can be expected that the administration of these cytokines will cause an increase in the number of MHC molecules and CEA complexes on tumor cells as a secondary effect of the adjuvant cytokines described above. The tumor cells should then be severely affected by CTL-mediated cytotoxicity.

In conclusion, treatment with DCs pulsed with CEA652 with the adjuvant use of IFN-α and TNF-α was found to be a safe procedure. Clinical and immunological responses were observed in two patients out of seven in the adjuvant arm, in contrast to no response in the patients not receiving adjuvant cytokines. These findings may indicate that CEA652 pulsed DCs are immunogenic and a feasible treatment option for solid cancer patients and that the adjuvant cytokines IFN-α and TNF-α are likely candidates for DC-based vaccine therapy although their effects require further statistical investigation.

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