Immunotherapy of Autologous Tumor Lysate-loaded Dendritic Cell Vaccines by a Closed-flow Electroporation System for Solid Tumors

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Abstract. *Dendritic cell (DC)-based vaccines with the use of various antigen loading methods have been developed for cancer immunotherapy. Electroporation (EP) of a whole tumor cell lysate into DCs was previously found to be more potent for eliciting antigen-specific CD8 + T-cells compared to co-incubation of tumor cell lysates with DCs in vitro. In the present report, we studied the feasibility, safety and antitumor effect in the clinical use of an EP-DC vaccine for the immunotherapy of various types of human solid tumors. We successfully prepared an autologous tumor lysate-loaded EP-DC vaccine with high cell viability by the closed-flow electroporation system. In the phase I clinical trial, mild adverse events associated with the EP-DC vaccine were found during the treatment of advanced or recurrent cancer, or during the adjuvant therapy of some types of cancer; no autoimmune responses were observed after treatment with the autologous tumor lysate-loaded EP-DC vaccines. For the antitumor effect of the EP-DC vaccine against the 41 various types of solid tumor, the overall response rate [complete remission (CR) + partial response (PR)] was 4.9% (2/41) and the clinical benefit rate [CR+ PR + long stable disease (SD)] was 31.7% (13/41). Furthermore, the delayed-type hypersensitivity (DTH) reactivity was positive in most cases of long SD and the positive rate of DTH was 91.7% (11/12) for the patients with clinical benefit. In conclusion, the safety and feasibility of the EP-DC vaccine with autologous tumor lysates were confirmed, and it was found that the antitumor effect might be associated with the immunological response induced by the EP-DC vaccine for cancer immunotherapy.*

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Recently, the development of vaccine therapy for cancer has progressed at a rapid pace. The main reasons for this are studies on tumor-specific antigens carried out and reported for the past 20 years, and the fact that details of the immune system in connection with innate and acquired immunity have already been elucidated, as represented by the Nobel Prize for Physiology or Medicine in 2011. In particular, rapid progress has been accomplished owing to the identification of the CD8 + cytotoxic T-lymphocyte (CTL) inductivity epitope peptide in the 1990s (1), such as the melanoma antigen, and the development of research on dendritic cells (DCs) as antigen-presenting cells (APCs). DCs are well-known as being the strongest and the only APCs that induce the overexpression of the major histocompatibility complex (MHC) and co-stimulator molecules, such as CD80 (B7-1) and CD86 (B7-2), and also stimulate naive T-cells before sensitization from antigens (2).

In 1994, Sallusto and Lanzavecchia revealed that peripheral blood mononuclear cells (PBMCs) cultured with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) can induce the differentiation and induction of DCs (3). The methods of *ex vivo* DC preparation facilitated clinical studies on cancer vaccines with combination between DCs and various types of cancer antigens. The morphologies of tumor-specific antigens such as apoptotic cells, proteins, mRNAs, and peptides as tumor antigens were studied (4-7). An antitumor peptide composed of 9-15 amino acids was presented on MHC class I molecules on the DC surface. Tumor-specific CD8 + CTLs are effectively stimulated and induced by recognition *via* MHC class I molecules with co-stimulator molecules. On the other hand, by using a protein antigen or mRNA, it was found that the presentation is performed *via* MHC class I and class II molecules, and the presentation changes depending on the method of delivering the antigen to DCs through processing inside the DCs (8, 9). Previously, we studied

electroporation as a way to deliver an antigen to DCs with high cell viability. It was also clearly shown that the antigen-specific CD8 + CTLs were induced effectively by electroloading of the antigen to DCs as compared with co-culture of the antigen with immature DCs (10).

In this study, we examined the safety and efficacy of the electroporation DC vaccine (EP-DC) in which tumor antigens were electroloaded to DCs using an autologous tumor lysate solubilized from surgically-resected tumor tissues.

Patients and Methods

Collection and culture of dendritic cells. PBMCs were collected by leukapheresis from patients with cancer for 2-3 hours and purified with Ficoll. PBMCs were cultured in an incubator at 37˚C with 5% CO_2. The adherent cell fraction was used for the DC culture by incubation for five days in AIM-V (Life Technologies, Carlsbad, CA, USA) medium supplemented with IL4 and GM-CSF. For maturation, DCs were cultured for an additional two days in AIM-V medium supplemented with GM-CSF, IL4, tumor necrosis factor alpha (TNFα), and prostaglandin E2 (Sigma-Aldrich, St. Louis, MO, USA).

Tumor lysates. To prepare autologous tumor lysates, the specimen from a surgically-resected tumor was cleaned of nonmalignant tissues with a scalpel and was lysed by passage through three freeze/thaw cycles and sonication at 4˚C. The tumor lysate was centrifuged and the supernatants were passed through a 0.22 μm filter (Millipore Corporation, MA, USA). The protein content of the lysates was determined using BCA assay (Thermo Fisher SCIENTIFIC, Rockford, IL, USA) and aliquots were stored at –80˚C until use.

Electroporation of DC vaccines. Electroporation was performed using MaxCyte CL-2 Processing Assembly and MaxCyte GT electroporation system (MaxCyte, Inc, Gaithersburg, MD, USA), as previously described (10). The autologous tumor lysate was electroloaded to DCs collected from patients with various malignancies in a closed-flow electroporation system. After electroporation, the viability of EP-DCs was examined for all patients. Sterility and endotoxin levels were also examined for each EP-DC.

Phase I clinical trial. The phase I clinical trial, which was approved by the research ethics committee of the Seta Clinic (approval number: SCG08028), was performed to assess the safety and feasibility of the EP-DC vaccine with the use of the autologous tumor lysates. Inclusion criteria in this study were as follows: i) the resected tumor tissue was diagnosed as malignant, ii) the patient was over 20 years old, iii) the collected lysate from the tumor tissue was more than 5 mg, iv) the performance status of the patient was 0 or 1, and v) the cell number of the DC vaccine was more than 5×10^6. The phase I trial involved 10 patients with advanced malignancies, and PBMCs were collected from each patient by the leukapheresis protocol. The DC vaccine was given to each patient six times at 2-week intervals. As the primary endpoint, adverse events were monitored on the basis of Common Terminology Criteria of Adverse Events (CTCAE) ver. 3.0 (11). Delayed-type hypersensitivity (DTH) reactivity was determined on the forearm after each vaccination. The DTH reaction was evaluated as positive when the diameter of the indurations was larger than 5 mm diameter or the skin redness was larger than 10 mm diameter.

Antitumor effect of EP-DC vaccine. After its safety was confirmed, the EP-DC vaccine with tumor lysate was administered to the patients from whom the autologous tumor lysate has been successfully prepared. After obtaining the informed consent of patients with the advanced or recurrent cancer at the Seta Clinic, the antitumor effect of the EP-DC vaccine was evaluated on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST) (12) with imaging, such as computed tomography (CT), magnetic resonance image (MRI), or positron emission tomography (PET)/CT. Furthermore, the DTH reaction observed either before the first, third, fourth, or fifth vaccination in correlation with RECIST was also evaluated. Additionally, safety and feasibility were also monitored for all the patients, including the patients with progressive recurring cancer and those subjected to adjuvant therapy.

Statistical analysis. Fisher's exact test was performed to evaluate the significance the between antitumor effect and DTH reactivity.

Results

Patients' characteristics in phase I clinical trial. Three patients with advanced or recurrent cancer and seven being treated with adjuvant therapy were enrolled in this study before the resection of malignant tumor. Although two out of the seven patients were enrolled provisionally as being on adjuvant therapy, they were finally registered as patients with advanced cancer, because tumor remnants were confirmed at the start of the EP-DC vaccination. Patients' characteristics are shown in Table I. Various malignancies were registered for this clinical trial, including lung, liver, and colorectal cancer and sarcomas. Resected tumor tissues included three from primary lesions and seven from recurrent sites. Soluble tumor lysates were successfully prepared (more than 5 mg of protein), and each tumor lysate was electroloaded into immature DCs using MaxCyte GT. DCs were collected from the patients by leukapheresis, and no severe adverse events were observed during or after the leukapheresis. The total number of collected DCs was from 2.5×10^7 to 16.4×10^7, and the average cell number was 10×10^6 (range from 3.9×10^6 to 27.4×10^6) for each vaccination.

DTH reactivity and feasibility. Six vaccinations using the autologous tumor lysate-loaded EP-DC vaccine were given to each patient in this study. Skin tests were evaluated before treatment and at each vaccination. The DTH reactivity was evaluated for 10 patients; eight showed a positive result and two showed a negative result. Safety as the primary endpoint was evaluated by CTCAE ver. 3.0 and a total of 11 adverse events were observed for eight patients (Table I). However, for three patients, only four events were considered to be associated with EP-DC vaccination, such as fever (grade 1), rash (grade 2), and fatigue (grade 1 or 2). Additionally,

Table I. *Characteristics of patients with various solid tumor types in phase I clinical trial of autologous tumor lysate-loaded electroporation dendritic cell (EP-DC) vaccine. Adverse events were monitored as the primary endpoint, as well as the total injected number of EP-DC and DTH reactivity.*

No	Age, years/ Gender	Diagnosis	Residual tumor	Combination therapy	Average no. of DCs administered	Adverse event associated with EP-DC vaccine	Adverse event not associated with EP-DC vaccine
1	60/M	Ureteral cancer	+	-	5.3×10^6	N/A	N/A
2	38/F	Clear cell sarcoma	-	-	9.4×10^6	Rash (Gr2)	Anemia (Gr1)
3	63/M	Colorectal cancer	-	-	11.8×10^6	Fatigue (Gr2)	N/A
4	69/M	MFH	+	-	3.9×10^6	Fatigue (Gr1) Fever (Gr1)	N/A
5	65/M	Colorectal cancer	+	FOLFOX	8.3×10^6	N/A	Stomatitis (Gr1)
6	57/M	Tongue cancer	-	-	11.5×10^6	Injection site reaction (Gr1)	Mucositis (Gr1)
7	73/M	Bladder cancer	-	-	27.4×10^6	N/A	Anemia (Gr1)
8	64/F	Lung cancer	+	RT	7.7×10^6	N/A	Dizziness (Gr1)
9	44/M	Liver cancer	-	-	11.4×10^6	N/A	N/A
10	62/M	Glioma	+	Temozolomide IFN-β	10.8×10^6	N/A	Dizziness (Gr1)

MFH: Malignant fibrous histiocytoma; FOLFOX: chemotherapy regimen using fluorouracil folinic acid and oxaliplatin; RT: radiotherapy; IFN-β: interferon-beta; N/A: not applicable; Gr: grade.

injection site redness (grade 1) was exhibited around the vaccination site as a localized reaction in one patient. Hematological toxicity related to the EP-DC vaccine was not observed.

Patient backgrounds in clinical use of EP-DC vaccine. After the phase I clinical trial of the EP-DC vaccine was performed, 52 patients with solid tumors were additionally registered for clinical use of the EP-DC vaccine for five years from 2008 to 2012 (Table II). The age range was from 19-78 years (median age was 54 years); the numbers of males and females were 16 and 36, respectively. Forty-one of the 52 patients had progressive or recurrent cancer, and 11 patients were administered the EP-DC vaccine as adjuvant therapy after surgery. Forty-two out of the 52 patients received other treatments around the same time as the EP-DC vaccination, whereas the other 10 patients were treated only with the autologous tumor lysate-loaded EP-DC vaccine. For 37 patients, chemotherapy or molecular-targeting therapy was given in combination with the EP-DC vaccine. Four patients with breast cancer received both EP-DC vaccination and hormone therapy. Interferon-alpha (IFNα) therapy was given to one patient with renal cell cancer in addition to the EP-DC vaccination. The resection of tumor tissues included 19 primary lesions and 33 recurrent sites for the preparation of autologous tumor lysates. To prepare tumor lysates for the EP-DC vaccine, we extracted more than 5 mg of protein lysate from tumor tissues for all the patients. There were no severe organ toxicities or autoimmune responses in the 52 patients in this study (data not shown).

Antitumor effect of the EP-DC vaccine on solid tumors. We evaluated the antitumor effect of the EP-DC vaccine with the use of RECIST criteria only for the 41 patients with advanced or recurrent cancer with residual tumor after surgical resection. The antitumor effect of the autologous tumor lysate-loaded EP-DC vaccine is shown in Table III. Moreover, the clinical responses of the 41 patients with advanced or recurrent tumors in this study revealed 0 complete remission (CR), two partial responses (PR), 11 long stable diseases (SD) (more than 24 weeks), four SD, and 22 progressive diseases (PD) by the RECIST criteria. For two cases, the antitumor effect of the EP-DC vaccine was not evaluable (NE) with imaging. The overall response rate was 4.9% and the clinical benefit rate was 31.7%. One of the two cases of PR was a patient with renal cell carcinoma treated with a combination of molecular target drug, although the DTH reactivity for this patient was negative. Another case of PR was of breast carcinoma; this patient received both the EP-DC vaccine and hormone therapy, and the DTH reactivity was positive. The DTH reaction was found to be positive for 31 patients, negative for six patients, and the remaining four patients, were not examined. For the cases of PR, long SD, SD, and PD, the positive rates of DTH reactivity were 1/2, 10/10, 2/4, and 18/21, respectively. There were no significant differences between the antitumor effect and DTH reactivity for the EP-DC vaccine for patients with advanced or recurrent cancer in this study, however, the positive rate for DTH was 11/12 for patients with clinical benefit (PR + long SD).

Discussion

DCs are the most important of all APCs because they can process and present antigens for the stimulation of primary and secondary T-cell responses (13, 15). Various DC-based vaccines have been employed in clinical trials of cancer immunotherapy. In 2010, the U.S. Food and Drug

Table II. *Characteristics of 52 patients in clinical use of autologous tumor lysate-loaded electroporation dendritic cell (EP-DC) vaccine.*

Characteristic	No. of patients
Age, years (Median-54)	19-78
Gender	
Male	16
Female	36
Primary site	
Lung	8
Colorectum	7
Breast	6
Stomach	4
Liver	3
Brain	2
Esophagus	2
Tongue	2
Kidney	2
Bile duct	2
Sarcoma	2
Other	8
Residual tumor	
Yes	41
No	11
Combined therapy	
Chemotherapy/molecular-targeted drug	37
Hormone therapy	4
Cytokine therapy	1
None	10
Tumor lysate source	
Primary site	19
Metastasis	33

Table III. *Clinical response and delayed-type hypersensitivity (DTH) reactivity to autologous tumor lysate-loaded electroporation dendritic cell (EP-DC) vaccine for the 41 patients with advanced or recurrent solid tumors.*

Clinical response	DTH reactions			
	Positive	Negative	NE	Positive rate (%)
CR (n=0)	0	0	-	-
PR (n=2)	1	1	-	1/2 (50.0)
Long SD (n=11)	10	0	1	10/10 (100.0)
SD (n=4)	2	2	-	2/4 (50.0)
PD (n=22)	18	3	1	18/21 (85.7)
NE (n=2)	-	-	2	-
Total	31	6	4	31/37 (83.9)

CR: Complete remission; PR: partial response; long SD: long stable disease; SD: stable disease; PD: progressive disease; NE: not evaluable.

Administration (FDA) approved sipuleucel-T (Provenge®; Dendreon Corp., Seattle, WA, USA), an autologous cellular immunotherapy for the treatment of metastatic hormone-refractory prostate cancer (15). For sipuleucel-T, APCs are obtained by leukapheresis and are cultured with a recombinant fusion protein. Although the precise mechanism is unknown, the APCs activated by recombinant human protein are returned to the patients to stimulate potentially the effector T-cells (16, 17). For DC-based vaccines, various tumor antigens are loaded onto DCs. In clinical trials, it was reported that autologous glioma lysate DC vaccination was associated with wider patient eligibility compared with glioma-associated peptide DC vaccination (18). Because of human leukocyte antigen (HLA) allele restrictions on the peptide-DC trial, only a few screened patients were eligible for treatment, whereas most of the patients passed the eligibility screening for the autologous tumor lysate-DC trial. For the present autologous tumor lysate-loaded EP-DC vaccine, most of the patients were able to receive EP-DC vaccine therapy if a sufficient amount of autologous tumor lysates was prepared.

Processing of exogenous tumor lysates by DCs leads predominantly to the presentation of peptides on MHC class II molecules, although co-loading of autologous tumor lysates can be easily accomplished with minimal additional processing or safety risk (19). We previously reported that loading human DCs with tumor cell lysates using EP could potentially be superior to the current practice of lysate co-incubation *in vitro* (10). In this report, we studied the safety and feasibility of the tumor lysate-loaded EP-DC vaccine in phase I clinical trials and also evaluated the antitumor effects of the EP-DC vaccine on the treatment of human solid tumors.

In the phase I clinical trial with the therapeutic use of the autologous tumor lysate-loaded EP-DC vaccine for malignant tumors, 10 patients were enrolled, five advanced or recurrent cases and five postoperative adjuvant cases after the resection of tumors. Previously, we produced EP-DC vaccines while maintaining high cell viability post-EP. Similarly, in the present trial, 9 out of 10 patients were successfully administered the tumor lysate-loaded EP-DC vaccine with more than 90% cell viability (data not shown). For patients administered the tumor lysate-loaded DC vaccine, various toxicities were reported but they were not severe, such as fatigue, eruption, and transient redness or swelling at the injection sites (20, 21). Among 10 patients in the phase I trial and additional 52 patients treated with tumor lysate-loaded EP-DC vaccine, there were no severe adverse events beyond grade 2, neither for hematological, nor for non-hematological toxicities, All 62 patients received their vaccinations in an outpatient setting.

Some investigators described that there is no relationship between the antitumor effect and immunological response, such as CTL induction, IFNγ expression, or DTH reactivity, although Barth *et al*. reported that tumor-specific responses were associated with improved survival of patients

administered DC vaccines activated with CD40L (22). In this study, a positive DTH response was found in 83.9% of the 37 patients with advanced or recurrent cancer with the clinical use of the tumor lysate-loaded EP-DC vaccine. There was no significant correlation between the antitumor effect and DTH because of the high positive rate of DTH reactivity. However, positive DTH responses did appear to be essential for patients with PR and long SD, except for one case of PR without DTH response. In this case, the clinical outcome was associated more with the treatment using the molecular-targeted drug, sunitinib, for recurrent renal cell cancer, rather than with the EP-DC vaccine. For the DC-based cancer vaccine, there are some reports that describe lack of clinical response despite good immunological responses, because the immune competence of patients with large tumor burdens was diminished. Therefore, it might be necessary to evaluate the immunological competence of patients before treatment, as well as to carry out immunomonitoring during EP-DC vaccination.

One potential disadvantage of the use of tumor lysates is the inclusion of self-antigens that could lead to the generation of an autoimmune response. In most of the trials for DC-based cancer vaccines with tumor lysates, no notable organ toxicity or autoimmune response was identified, similarly to the findings of the present study, although autoimmune toxicity associated with the DC vaccines was reported in a melanoma vaccine trial (23). Recently, improvement of the global immune dysfunction has been shown to be important, especially immune suppression by regulatory T-cells or myeloid-derived suppressor cells. Antibodies against CTL-associated antigen-4 (CTLA4) or programmed death-1 (PD1) are clinically used for melanoma, although autoimmune toxicity is one of the major side-effects of these antibodies (24, 25). Ribas *et al*. reported that the combination of melanoma antigen recognized by T-cells 1 (MART1) peptide-pulsed DCs and CTLA4-blocking antibodies results in objective and durable tumor responses at the high end of the range of the response rate expected with either agent alone (26). It is important to record adverse events and autoimmune responses carefully with the use of both the autologous tumor lysate-loaded EP-DC vaccine and such antibodies in order to break the immune suppression in patients with cancer.

In conclusion, safety and feasibility were confirmed for the EP-DC vaccine with autologous tumor lysate in a phase I clinical study and for clinical use in combination with standard drug therapy. An immunological response was potentially associated with the antitumor effect of the EP-DC vaccine; however, there was no significant relationship between the DTH reactivity and clinical response. In the future, the correlation of host immune competence and clinical outcomes should be studied. Additionally, the combined therapy of the EP-DC vaccine and CTLA4 or PD1 antibodies in order to break the immunosuppression induced by various malignancies, should be investigated.

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