Immune responses in patients with esophageal cancer treated with SART1 peptide-pulsed dendritic cell vaccine

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Abstract. Patients with advanced stage of squamous cell carcinoma of esophagus have a poor prognosis with a lethal outcome. In order to explore the feasibility and effectiveness of dendritic cell (DC)-based immunotherapy for squamous cell carcinoma of esophagus, we performed a phase I/II clinical trial of monocyte-derived dendritic cells (moDCs) pulsed with SART1 peptide in seven patients with advanced stage of this disease. Although the feasibility of this therapy was definite, the effectiveness was not clearly confirmed in advanced stage of squamous cell carcinoma of esophagus. However, in vitro study revealed that moDCs generated for this therapy possessed a potent ability of inducing SART1 peptide-specific cytotoxic T lymphocytes (CTLs). In addition, these moDCs were demonstrated to be able to produce exosomes with an antigen presenting ability for inducing SART1 peptide-specific CTLs. ELISPOT assay using cryopreserved patient's lymphocytes demonstrated that IFN-γ ELISPOTs were increased after four times of SART1 peptide-pulsed moDC vaccinations compared with before the vaccination in a patient. The present study demonstrated that moDCs prepared from advanced stage of squamous cell carcinoma of esophagus possess a good immune function and in vivo immune responses (detected by ELISPOT assay) were evoked by the infusion of these moDCs. These findings suggest that DC-based immunotherapy could be one of the modalities applicable for squamous cell carcinoma of esophagus.

Introduction

In Japan, >90% of esophageal cancer is squamous cell carcinoma, which prognosis is poorer than adenocarcinoma of esophagus (1). Patients with advanced stage of esophageal cancer have a poor prognosis with a lethal outcome, despite efforts to improve diagnostic procedures and treatment modalities (2).

Dendritic cells (DCs), which are potent antigen-presenting cells, could coordinate innate and adaptive immune responses. Hence, DC-based tumor immunotherapy was introduced in the patients with prostate cancer, melanoma, renal cell carcinoma, glioma, gastric cancer, colon cancer, and pediatric solid tumor (3). In 2010, monocyte-derived dendritic cells (moDCs) pulsed with fusion antigen protein consisting of full-length prostatic acid phosphatase (PAP) and full-length GM-CSF were approved by U.S. Food and Drug Administration for the treatment of men with hormone refractory prostate cancer. Cellular immunotherapy using these moDCs prolonged overall survival among men enrolled in the phase III clinical study compared with placebo group (4). As demonstrated in phase III trials of this prostate cancer immunotherapy, moDC-based cellular immunotherapy was shown to be effective in cancer patients when the candidate is selected properly. However, with regard to DC-based cellular immunotherapy for carcinoma of esophagus, only few studies (5-7) have been carried out. One of the studies dealt with WT1 peptide-pulsed DC therapy with activated T lymphocyte therapy for advanced cancers including two patients with esophageal cancer (5). The study showed that there is beneficial effect to some extent. The other two reports dealt with DC therapy for primary malignant melanoma of the esophagus (6,7). On this note, Asakage et al showed that tumor lysate-pulsed DC therapy is a safe and promising approach as adjuvant therapy for primary malignant melanoma of the esophagus (6). Likewise, Ueda et al reported that peptide-specific immune response could be induced in patients with primary malignant melanoma of the esophagus after immunotherapy using DCs pulsed with MAGE peptides (7).
We performed phase I/II clinical trial of moDCs pulsed with SART1 (8) peptide for patients with advanced squamous cell carcinoma of esophagus. In addition, we performed in vitro studies concerning cytotoxicity of patient's lymphocytes cultured with SART1 peptide-pulsed moDCs or exosomes secreted from these moDCs against esophageal carcinoma cell line. We also performed an IFN-γ ELISPOT assay using patient lymphocytes obtained before and after SART1 peptide-pulsed moDC vaccination. Although clinical benefit was not clearly demonstrated, in vitro and in vivo immune responses caused by SART1 peptide-pulsed moDC vaccination was revealed in the present study.

Materials and methods

Study design. The study was carried out according to a protocol approved by the Institutional Review Board of Niigata University School of Medicine and conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all patients. Seven patients were enrolled in this open-labeled, non-randomized phase I/II clinical trial. The primary aim of the study was to evaluate feasibility and safety, whereas the secondary aim was to evaluate immunological and clinical responses to DC vaccination (Table I).

Patients and treatment. Eligibility criteria for the present study were as follows: i) advanced or relapsed squamous cell carcinoma of esophagus, which had been treated with standard therapy; ii) presence of HLA-A*24:02; and iii) performance status (PS) ≤1 (ECOG-scale).

Theuffy coat cells of patients were collected by leukapheresis (in 14 out of 21 times DC preparation) or bag method (in seven times) with written informed consent. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque (Lymphoprep; Axis-Shield Poc AS, Oslo, Norway) density centrifugation. Monocytes were isolated by culturing PBMCs in plastic culture dish (BD Biosciences, San Jose, CA, USA) at a cell concentration of 3–5x10⁶/ml and removing non-adherent cells from the dish. Immature moDCs were induced from monocytes by culturing plastic adherent cells in the same culture dish containing RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) with 5% autologous serum, 100 ng/ml GM-CSF (Kirin Brewery Co., Ltd., Gunma, Japan) and 10 ng/ml IL-4 (Schering-Plough Research Institute, Kenilworth, NJ, USA) for 7 days. Immature moDCs were matured by adding 10 ng/ml TNF-α (Peprotech, Inc., Rocky Hill, NJ, USA) on day 6. Mature moDCs were collected from culture dish by pipetting and occasionally using cell scraper (Corning Life Sciences, Tewksbury, MA, USA).

Tumor antigen peptide used for the study was SART1(kon.top) (EYRGFTQDF; HLA-A*24:02 restricted, GMP grade), which was produced by Multiple Peptide Systems (San Diego, CA, USA) and donated by Prof. Kyogo Itoh (Kurume University, Fukuoka, Japan). SART1 peptide was added to the moDC culture at a concentration of 50 µg/ml during the last 24 h. In the last three patients, keyhole limpet hemocyanin (KLH; carrier protein for peptide antigen) (Calbiochem, La Jolla, CA, USA) was pulsed at a concentration of 50 µg/ml together with SART1 peptide for the last 24 h of moDC culture (Table I). On occasion, PBMCs and peptide-pulsed moDCs were cryopreserved for later in vitro study.

On the day of vaccination, peptide-pulsed moDCs were washed and re-suspended in 500 µl saline with 5% autologous serum and transferred to a 1 ml syringe for injection. The moDC suspension was injected intravenously (IV) in the first four patients (patients 1–4) and subcutaneously (SC) in the upper arm in the last three patients (patients 5–7). Infusions with peptide-pulsed moDCs were repeated every three weeks up to five times depending on the patient.

Clinical evaluation. Evaluation with CT scan and clinical examinations were performed before, during and after vaccinations. A skin test for delayed-type hypersensitivity (DTH) reaction was performed using an intradermal injection of 100 µl of the peptide or KLH (5 mg/ml each) on the palmar side of the forearm. Saline solution (100 µl) was used as a negative control. More than 2 mm red induration area after 48 h was defined as a positive DTH skin test reaction.

Analysis of surface phenotypes of moDCs prepared for infusion. Immediately before injection, antigen-pulsed moDCs were spared and subjected to phenotypic analysis as previously described (9). The cells were stained by incubation with monoclonal antibodies against CD1a (Immunotech, Marseille, France), CD14, CD80, CD86 and HLA-DR (BD Biosciences) together with the relevant isotype controls to analyze the expression of cell surface antigens.

Preparation of moDC-derived exosomes. moDC-derived exosomes were prepared using the method described by Zitvogel et al (10). The whole culture medium of SART1-pulsed moDCs was harvested at the time of preparing moDCs for injection. moDC culture medium was centrifuged at 300 x g for 20 min and the supernatant was collected. The supernatant was centrifuged at 10,000 x g for 30 min and the supernatant was collected again for eliminating cell debris. Then the supernatant was ultra-centrifuged at 100,000 x g for 60 min and the pellet was collected and washed once in a large volume of medium. Exosome pellet was finally dissolved at the concentration of exosomes derived from 10⁷ moDCs in 1 ml RPMI-1640 medium. The protein concentration in the exosome preparation was measured and used for SART1 peptide-specific cytotoxic T lymphocyte (CTL) induction assay.

Identification of exosome. Exosome was identified by demonstrating the expressions of both HLA-DR and CD86 on the surface of the nanoscale vesicle (Fig. 1) (11). Anti-PE microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach Germany) were incubated with PE labeled anti-HLA-DR monoclonal antibody (BD Biosciences) for 1 h at 4°C with tapping every 10 min. The mixture was washed with PBS by centrifuging at 9,100 x g (10,000 rpm) for 10 min at 4°C twice in order to eliminate free monoclonal antibody. Microbeads pellet was suspended with Fc receptor blocking solution (PBS with 0.5% human γ-globulin and 0.1% sodium azide) (for blocking the Fc receptors possibly expressed on the surface of exosome) and mixed with exosome solution, and then incubated for 1.5 h at 4°C with tapping every 20 min. The mixture was washed...
Table I. Patient profile, mode of DC therapy and clinical response.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/Gender</th>
<th>Tumor progression</th>
<th>Previous therapy</th>
<th>Cytokines for DC culture</th>
<th>Antigen peptides</th>
<th>Mode of infusion</th>
<th>No. of infusion</th>
<th>Average no. of infused cells (x10⁸)</th>
<th>DTH</th>
<th>Effect of DC therapy</th>
<th>Tumor marker</th>
<th>Outcome</th>
<th>Adverse effects (association)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58/M</td>
<td>Liver metastasis</td>
<td>NAC, esophagectomy, hepatectomy</td>
<td>GM-CSF, IL-4</td>
<td>SART-1</td>
<td>IV</td>
<td>3</td>
<td>3.08 ND</td>
<td>PD</td>
<td>Elevated</td>
<td>Elevated</td>
<td>Died</td>
<td>Died (10 M)</td>
</tr>
<tr>
<td>2</td>
<td>71/M</td>
<td>Lung metastasis</td>
<td>Esophagectomy, Cx, Rx</td>
<td>GM-CSF, IL-4</td>
<td>SART-1</td>
<td>IV</td>
<td>5</td>
<td>1.28 ND</td>
<td>NC</td>
<td>Negative</td>
<td>Survived</td>
<td>Survived</td>
<td>Hypophosphatemia (possible)</td>
</tr>
<tr>
<td>3</td>
<td>61/M</td>
<td>Aortic invasion, liver and lung metastasis</td>
<td>Cx</td>
<td>GM-CSF</td>
<td>SART-1</td>
<td>IL-4</td>
<td>IV</td>
<td>3</td>
<td>1.79 ND</td>
<td>PD</td>
<td>Elevated</td>
<td>Died (2 M)</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>59/M</td>
<td>Mediastinal LNs metastasis, pleural infiltration</td>
<td>Esophagectomy, Cx, Rx</td>
<td>GM-CSF, IL-4</td>
<td>SART-1</td>
<td>IV</td>
<td>2</td>
<td>0.54 ND</td>
<td>PD</td>
<td>Elevated</td>
<td>Died (1 M)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>66/M</td>
<td>Liver, kidney and skin involvement</td>
<td>NAC, esophagectomy</td>
<td>GM-CSF, IL-4, TNF-α</td>
<td>SART-1</td>
<td>SC</td>
<td>3</td>
<td>1.09 Negative</td>
<td>PD</td>
<td>Elevated</td>
<td>Died (2 M)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>67/M</td>
<td>Liver and neck LNs metastasis</td>
<td>Esophagectomy, Rx</td>
<td>GM-CSF, IL-4, TNF-α</td>
<td>SART-1</td>
<td>SC</td>
<td>3</td>
<td>0.31 KLH positive</td>
<td>PD</td>
<td>Elevated</td>
<td>Died (4 M)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>53/M</td>
<td>Liver and abdominal LNs metastasis</td>
<td>NAC, esophagectomy, Cx</td>
<td>GM-CSF, IL-4, TNF-α</td>
<td>SART-1</td>
<td>SC</td>
<td>2</td>
<td>0.77 KLH positive</td>
<td>PD</td>
<td>Elevated</td>
<td>Died (3 M)</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

DC, dendritic cell; NAC, neoadjuvant chemotherapy; Cx, chemotherapy; Rx, radiotherapy; KLH, keyhole limpet hemocyanin; IV, intravenously; SC, subcutaneously; DTH, delayed-type hypersensitivity; ND, not done; PD, progressive disease; NC, no change.
lymphocytes in moDc-treated patients. Briefly, S art1 irradiated with 30 g y of l37cs generated from gamma irradiated peptide-pulsed moDcs, which were used for vaccination, were for evaluating an antigen-specific proliferative capacity of Proliferation assay was performed. Proliferation assay.

Cytotoxicity assay. Patient lymphocytes, which were cultured with SART1 peptide-pulsed autologous moDcs or their exosomes for 4 weeks, were used as effector cells in 

ELISPOT assay. Cryopreserved patients' PB-MNCs were plated in 2 ml/well at the concentration of 2x10^6 cells in 24-well plates (BD Biosciences) in 5% human serum-containing RPMI-1640 medium with 10 µg/ml of SART1 peptide. Two days later, 300 IU/ml IL-2 was added to the cultures. The cultured cells were tested for reactivity in the ELISPOT on day 12. The ELISPOT assay for quantifying SART1 peptide-specific IFN-γ-releasing cells was performed using ELISpot [13] for Human IFN-γ kit (Mabtech AB, Nacka Strand, Sweden). The cultured cells and SART1 peptides were added to the ELISPOT plates, which had been coated with anti-IFN-γ antibody (I-DIK), and the plates were incubated overnight. The following day, biotinylated detection antibody (7-B6-1-biotin) was added to the washed wells. The plates were incubated for 2 h and washed, and the streptavidin-ALP was added to each well. Plates were incubated at room temperature for 1 h, and the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT-plus) was added to each well and incubated at room temperature for 15 min.
The spots were counted using stereomicroscope imaging system (Olympus Corp., Tokyo, Japan), and the peptide-specific CTL frequency could be calculated from the numbers of spot-forming cells.

**Statistical analysis.** The statistical relevance of differences in $^3$H-thymidine incorporation in proliferation assay and $^{32}$Cr release in cytotoxicity assay was evaluated with a two-way ANOVA, applying GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered as significant at $p<0.05$ and markedly significant at $p<0.01$.

**Results**

**Patient characteristics and vaccination with peptide-pulsed moDCs.** Seven patients with advanced stage of squamous cell carcinoma of esophagus were enrolled for the study. Patient characteristics are shown in Table I. The mean age of the patients was 62 years (range, 53-71 years) and all were males. All the patients, who had developed metastases to liver, lung or kidney and so on, were treated with operation, chemotherapy and/or radiotherapy before entering the clinical trial. In the first four patients, moDCs were prepared by culturing monocytes with GM-CSF/IL-4 and pulsing with SART1 peptide. In the last three patients, TNF-α was used for maturation of moDCs generated by culturing monocytes with GM-CSF/IL-4 and mature moDCs were pulsed with KLH in combination with SART1. Infusions of moDCs were undertaken two to five times, IV or SC in the first four patients, and in the last three patients, respectively.

The average number of infused cells in each infusion varied (0.31-3.08×10⁸ cells) from patient to patient depending on the method of blood drawing. Pre-culture number of MNCs, number of all infused cells, and percentage of large cells estimated by forward scatter and side scatter dot plots of flow cytometry, as well as the number of DCs (large cells) in each infusion of individual patient are shown in Table II. Comparison of these values among leukapheresis and bag method for blood drawing is shown in Table III. Mean and SD of moDCs in a single infusion procedure was 0.37±0.32×10⁸ cells in whole with 0.50±0.31×10⁸ cells in leukapheresis and 0.12±0.09×10⁸ cells in bag method (Table III).

**Surface phenotypes and allogeneic antigen presenting abilities of injected moDCs.** In the first four patients, immature moDCs were generated by culture with GM-CSF and IL-4 and pulsed with SART1 peptide only. In these patients, immature moDCs were used for the therapy since immature DCs were presumed to mature physiologically in the process of interaction with T cells in vivo. In the last three patients, mature moDCs were generated by culture with TNF-α in addition to GM-CSF and IL-4 and pulsed with SART1 peptide and KLH. These prepared moDCs were analyzed for surface phenotypes relating to antigen presentation. Although moDCs prepared from all the enrolled patients were positive for CD1a, CD80 CD86 and HLA-DR, the expression of CD83 was much higher in moDCs generated by culture with TNF-α in addition to GM-CSF and IL-4 compared with those generated by culture with GM-CSF and IL-4 only (Fig. 2). Therefore, the

| Table II. Number of infused cells each time of cell processing in patients with DC treatment. |
|-----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Patient no. | DC therapy no. | Pre-culture no. of MNCs (x10⁸) | No. of all infused cells (x10⁸) | % of large cells estimated by flow cytometry | Probable no. of DCs infused (x10⁸) |
| 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | | | | | | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | | | | | | | | |
| 4 | | | | | | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | | | | | | | | |

A Buffy coat cells were prepared by using 400 ml transfusion bag. Enough volume of blood could not be drawn by using leukapheresis. In all other cases, in DC therapy leukapheresis was used for blood drawing. DC, dendritic cell; nD, not done.
surface phenotypes of infused moDCs were characteristic for immature moDCs in the first four patients and mature moDCs in the last three patients. These moDCs were analyzed for antigen presenting ability by using allogeneic proliferation assay. Although mature moDCs showed slightly higher ³H-thymidine incorporation than immature moDCs in low stimulator/responder ratio of the proliferation assay, immature and mature moDCs were demonstrated to possess a considerable potent ability of antigen presentation (Fig. 3).

**DTH and effects of DC therapy.** Although skin DTH reactions against KLH were detected in two out of three patients vaccinated with moDCs pulsed with SART1 and KLH, DTH reaction against SART1 peptide was not observed in all the seven patients (Table I). One patient who received SART1 peptide-pulsed moDC vaccine (patient no. 2) remained stable for 20 months after moDC therapy judging from tumor marker and CT findings and he was categorized as no change (NC). But thereafter he developed lung metastasis, for which the operation was undertaken. The remaining six patients had progressive disease (PD) with the median survival of 3.7 months and no favorable response was observed during and after the vaccination course (Table I).

**Toxicity.** The vaccination was generally well-tolerated and no allergic reaction to the vaccine was observed. One patient who received SART1 peptide-pulsed moDCs showed a moderate

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**Table III. Comparison of number (mean ± SD) of infused cells among leukapheresis and the bag method.**

<table>
<thead>
<tr>
<th>Blood drawing</th>
<th>All (n=21)</th>
<th>Leukapheresis (n=13)</th>
<th>Bag (n=7)</th>
<th>Insufficient leukapheresis (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-culture no. of MNCs (x10⁶)</td>
<td>7.42±5.19</td>
<td>10.78±3.68</td>
<td>1.90±0.62</td>
<td>2.30</td>
</tr>
<tr>
<td>No. of all infused cells (x10⁶)</td>
<td>1.32±1.02</td>
<td>1.91±0.86</td>
<td>0.39±0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>% of large cells estimated by flow cytometry</td>
<td>24.9±14.36</td>
<td>25.6±12.8</td>
<td>27.6±15.9</td>
<td>3.40</td>
</tr>
<tr>
<td>Probable no. of DCs infused (x10⁸)</td>
<td>0.37±0.32</td>
<td>0.50±0.31</td>
<td>0.12±0.09</td>
<td>0.01</td>
</tr>
</tbody>
</table>

DC, dendritic cell.
hypophosphatemia, although the relationship with moDC vaccination was not definite (Table I).

**Induction of SART1-specific CTLs by using SART1 peptide-pulsed moDCs.** Lymphocytes of patient no. 7 primed with autologous SART1 peptide/KEYhole limpet hemocyanin (KLH)-pulsed moDCs three times showed a significant cytotoxicity against SART1 peptide-pulsed moDCs and KE4 cells, which were positive for the expression of both HLA-A24 and SART1, in an effector-to-target ratio dependent manner. However, CML-BC cell line C2F8 cells (13), which did not express SART1, were not killed by lymphocytes primed with SART1/KLH peptide-pulsed moDCs (Fig. 4).

**Increased reactivity of vaccinated patient’s lymphocytes against SART1 peptide/KLH-pulsed moDCs.** Reactivity of CD3+ T cells of patient no. 6 against moDCs pulsed with SART1 peptide/KLH was compared between CD3+ T cells in pre-treatment phase and those in post-vaccination phase (after three times infusion of SART1 peptide/KLH-pulsed moDCs). CD3+ T cells in post-vaccination phase showed a much higher reactivity against SART1 peptide/KLH-pulsed moDCs in autologous MLC compared with those in pre-treatment phase (Fig. 5). This enhancement of CD3+ T-cell reactivity

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**Figure 3.** Allogeneic antigen presenting ability of monocyte-derived dendritic cells (moDCs) prepared for DC-based immunotherapy. 3H-thymidine incorporation in proliferation assay performed by using moDCs generated from patient no. 1 or 6. Culture with GM-CSF/IL-4 for 7 days was used for generating moDCs in patient no. 1 and TNF-α was added for the last 24 h to GM-CSF/IL-4-induced moDCs in patient no. 6. These moDCs were pulsed with SART1 peptide or SART1 peptide plus keyhole limpet hemocyanin (KLH) during the last 24 h before the assay in patient no. 1 or 6, respectively. Red line shows cpm of 3H-thymidine incorporated by the stimulation with prepared moDCs and blue line by the stimulation with third party PBMCs. *H*-thymidine incorporations in patient no. 1 or 6 was similar to those obtained by using moDCs generated in patient nos. 1-4 or 5-7, respectively. **Markedly significant differences (p<0.01) between moDCs and PBMCs.

**Figure 4.** Induction of SART1-specific cytotoxic T lymphocytes (CTLs) using SART1 peptide-pulsed monocyte-derived dendritic cells (moDCs). PBMCs of patient no. 7, which had been primed in vitro with autologous SART1 peptide/keyhole limpet hemocyanin (KLH)-pulsed moDCs three times, were analyzed for their cytotoxicity against SART1 peptide-pulsed autologous moDCs, KE4 cells (esophageal cancer cell line with expression of both HLA-A24 and SART1) and C2F8 cells [chronic myelogenous leukemia-blastic crisis (CML-BC) cell line with no expression of SART1]. Cytotoxicity of CTLs was evaluated by percent release of 51Cr from target cells. **Markedly significant differences (p<0.01) between C2F8 and SART1 peptide-pulsed moDCs or KE4.

**Figure 5.** Increased reactivity of vaccinated patient lymphocytes against SART1 peptide/keyhole limpet hemocyanin (KLH)-pulsed monocyte-derived dendritic cells (moDCs). Reactivity of CD3+ T cells of patient no. 6 prepared at pre-treatment phase and those at post-vaccination phase (after three times therapy of SART1 peptide/KLH-pulsed moDCs) was investigated by performing proliferation assay using moDCs pulsed with SART1 peptide/KLH as stimulator cells. **Markedly significant differences (p<0.01) between CD3+ T cells at pre-Dc therapy phase and CD3+ T cells at post-Dc therapy phase.
was thought to be mainly caused by an increased reactivity against KLH.

Production of antigen-presenting exosomes by moDCs. Ultra-centrifuged preparation of supernatant from SART1-pulsed moDC culture in patient no. 1 and 2. Exosome pellet was dissolved at the concentration of exosomes derived from 10^7 moDCs in 1 ml RPMI-1640 medium. (A) For evaluating an allogeneic antigen-presenting ability of exosome solution, 100,000 allogeneic peripheral blood mononuclear cells (PBMCs) were cultured in 200 µl FBS-containing medium with 50 µl of exosome solution, which is equivalent to exosomes derived from 5x10^5 moDCs. (B) For evaluating an antigen-specific cytotoxic T lymphocyte (CTL)-inducing ability of these exosomes, autologous PBMCs were cultured in 2 ml autologous serum-containing medium with 500 µl of exosome solution, which is equivalent to exosomes derived from 5x10^6 moDCs. PBMCs cultured with exosomes for 4 weeks were used as effector cells in ^51Cr-release cytotoxicity assay using KE4 cells (HLA-A^*24/SART1^+) and allogeneic lymphocytes as target cells. Significant differences (p<0.05) between medium and exosome solutions from patient no. 1 or 2. Markedly significant differences (p<0.01) between exosome against KE4 and medium against KE4, medium against lymphocytes or exosome against lymphocytes.

SART1-specific T-cell response by ELISPOT. PBMCs obtained from three patients (patient no. 2, 3 and 4) before moDC vaccination and at time points during the vaccinations were analyzed for quantifying SART1 peptide-specific IFN-γ-releasing cells. One (patient no. 2) of three patients had a SART1-specific immune response in ELISPOT assay of lymphocytes at day 84 from the initiation of the vaccination (after four times of moDC vaccination) (Fig. 8). In the other two patients (patient nos. 3 and 4), SART1-specific IFN-γ-releasing cells did not increase probably due to the short period after the vaccination (not >42 days from the initiation of moDC vaccination). Patient no. 2, who showed a definite increase of IFN-γ ELISPOT after moDC vaccination, is the
Discussion

DC-based antitumor immunotherapy has been demonstrated to be feasible without side-effects and bring clinical benefits with immune responses (14), but esophageal cancers have been rarely enrolled in DC therapy so far. There have been several in vitro studies indicating DC immunotherapy as a promising strategy for esophageal cancer. Milano and Krishnadath reported a patient-specific autologous readout assay for pre-clinical testing of DC-mediated cytotoxic immune responses. They demonstrated that the use of DCs transfected with autologous total tumor RNA could be effective for treating esophageal cancer (16). While in the migration study of administered DCs, Fujiwara et al performed an intratumoral administration of in-labeled DCs in combination with preoperative chemotherapy in esophageal cancer patients. Their study revealed that the intratumoral administration of DCs during chemotherapy does not give rise to DC migration from the tumor to the draining lymph nodes, and suggested that an impairment of DC migration may be associated with difficulty in achieving an optimal clinical response in DC therapy (15). It is now generally recognized that clinical outcomes of patients receiving DC vaccination alone for advanced stage cancer have not been satisfactory. Therefore, the treatment strategy to combine DC therapy with another treatment modality to improve clinical outcomes is considered (16). With regard to antigen peptide-based immunotherapy for esophageal squamous cell carcinoma, Kono et al reported that the immune response induced by multiple-peptides vaccination could make the prognosis better by analyzing the data of a multicenter phase II clinical trial consisting of 60 patients with advanced stage of esophageal cancer (17).

Exosomes, which are nanoscale (50-100 nm) vesicles, can mediate an immune response by activating T lymphocytes (through antigen presentation), natural killer (NK) cells (through NKG2D ligand binding), and DCs (through antigen transfer) (18). Exosomes secreted by DCs loaded with tumor antigen have been shown to generate potent immune responses against cancer cells by inducing antigen-specific CD8+ T cells (19) and abolishing the suppressive function of regulatory T cells (20). Until now, only three clinical trials have been undertaken, on the application of exosomes for antitumor immunotherapy. Dai et al reported that autologous ascites-derived exosomes combined with GM-CSF could induce tumor antigen-specific CTL responses in phase I clinical trial for patients with colorectal cancer, with no to minimal adverse effects (21). Escudier et al disclosed that phase I clinical trial of autologous DC-derived exosomes was feasible and safe in patients with melanoma and minor to stable clinical responses were observed in skin and lymph node sites (22). Furthermore, Morse et al demonstrated a MAGE-specific T-cell response and increased NK lytic activity in patients with non-small cell lung carcinoma treated with autologous DC-derived exosomes loaded with multiple MAGE peptides (23).

Safety and efficacy were explored in the current phase I/II vaccination for patients with esophageal cancer. The vaccination was well-tolerated and no side-effect except for possible hypophosphatemia was observed, similar to those reported in other vaccination studies (24-26). One patient (patient no. 2) treated with SART1-pulsed mDCs remained stable for 20 months after mDC therapy, although thereafter he developed lung metastasis, for which surgery was undertaken. In patient no. 2, we could observe that the number of IFN-γ-producing cells increased after four times of SART1-pulsed mDC vaccination by IFN-γ ELISPOT assay. The other six patients died after 1-10 months from vaccination with PD. Although clinical and survival benefits were not observed in this vaccination treatment for the enrolled patients with advanced stage of squamous cell carcinoma of esophagus, feasibility of tumor antigen peptide-pulsed mDC therapy was demonstrated. In the present clinical trial, DTH against antigen peptide was negative, although positive for KLH in some patients. We used peptide itself for priming
in DTH. Instead of antigen peptides, antigen peptide-pulsed DCs should have been used for priming in DTH. On this note, Ellebæk et al reported that antigen-pulsed DCs should be used as antigen in DTH test in order to present antigens to obtain the highest local immune reaction (27). Also in vitro, the reactivity of patient’s CD3+ T cells against SART1 peptide/KLH-pulsed moDCs increased after three times DC vaccination. This enhancement of CD3+ T-cell reactivity was presumed to be due to an increased reactivity against KLH but not against SART1 peptide as shown in vivo of DTH. On the contrary, moDCs prepared from each patient expressed molecules associated with antigen presentation, such as CD1a, CD80, CD83, CD86 and HLA-DR, although the expression of CD83 among them was influenced by the culture method with or without TNF-α. Patient’s lymphocytes primed with SART1 peptide-pulsed moDCs were demonstrated to have a significant cytotoxic activity against HLA-A24+/SART1+ esophageal carcinoma cell line and SART1 peptide-pulsed autologous moDCs. These SART1 peptide-pulsed moDCs prepared from enrolled cancer patients were shown to produce antigen-presenting exosomes, which could generate SART1-specific CTLs in culture of autologous lymphocytes being stimulated with exosome preparation. In addition, ELISPOT assay using cryopreserved lymphocytes of the patients demonstrated that IFN-γ ELISPOTs were increased after four times of moDC vaccinations in one patient. These findings suggest that injected moDCs had an ability to induce antigen-specific CTLs and the patient lymphocytes acquired antigen-specific reactivity when primed with antigens presented by injected moDCs. In the present application of antigen peptide-pulsed moDCs for advanced stage of squamous cell carcinoma of esophagus and related in vitro and in vivo studies, it was shown that DC-based cellular immunotherapy for these cancer patients was feasible, functional DCs could be generated from these patients, and patient’s immunity is elevated by the infusion of DCs prepared from monocytes. In order to establish a clinically effective DC-based immunotherapy, the patient indication criteria for these therapies and the manner of preparing highly qualified DCs for injection were presumed to be the principle issues.

References


