Feasibility Study of Adoptive Immunotherapy for Metastatic Lung Tumors Using Peptide-pulsed Dendritic Cell-activated Killer (PDAK) Cells

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Abstract. We have established a novel culture system to generate effector lymphocytes designated as peptide-pulsed dendritic cell-activated killer (PDAK) cells using cultured dendritic cells (DCs), synthetic peptide, peripheral blood lymphocytes, and interleukin-2 plus immobilized anti-CD3 antibody. A feasibility study of an adoptive immunotherapy trial using PDAK cells was conducted on HLA-A2 and HLA-A24 cancer patients with antigen-positive lung metastasis that was defined by serological analysis or PCR analysis. Eleven patients with lung metastasis participated in the study: 6 with colorectal cancer, 2 with pancreatic cancer, 1 each with breast and lung cancer, and 1 with melanoma. The patients received either Muc-1, CEA, gp100, Her-2 or SART-3-PDAK cells generated in vitro, intravenously in combination with 350,000 U IL-2 weekly for 9 weeks, together with a planned dose-escalation schedule of three transfers each of 1×10⁷, 3×10⁷ and 1×10⁸ PDAK cells/kg for 6 patients, and with a uniform dose of 3×10⁷ PDAK cells/kg for the remaining 5 patients. Peptide/HLA-specific cytotoxic activity and TCRV' gene usage of PDAK cells were analyzed. All transfers of PDAK cells, which showed peptide/HLA-specific lysis, were well-tolerated in all patients, and adverse effects (elevation of transaminase, fever, and headache) were observed primarily at grade 1, but in no case greater than grade 2. The generation of sufficient cells to treat the patients with 3×10⁷ PDAK cells/kg was feasible using our culture system, but we were able to generate and administer the dose of 1×10⁸ PDAK cells/kg in only one patient. One partial response (PR) of lung metastasis occurred in a pancreatic cancer patient who received 3×10⁷ Muc-1-PDAK cells/kg. The cytolytic units of PDAK cells in this patient appeared to be substantially higher compared to those in PD patients. TCR gene usage analysis on PDAK cells revealed preferential usage of TCRVβ segments. These results suggest that adoptive immunotherapy using PDAK cells for cancer patients with antigen-positive lung metastasis is safe and feasible, and tumor response should be examined in a future clinical trial.

The discovery and molecular cloning of the crucial lymphocyte growth factor, interleukin-2 (IL-2) (1), has facilitated the clinical application of adoptive immunotherapy (AIT) for cancer using autologous lymphocytes activated in vitro with IL-2. Disease-associated immunosuppression in patients with cancer can disturb the effective emergence of anti-tumor responses in vivo (2). Therefore, the adoptive transfer of effector lymphocytes which have been educated and activated ex vivo to recognize tumor cells would, theoretically, provide an effective treatment for cancer. Of the techniques developed to date, the use of lymphokine-activated killer (LAK) cells (3), autolymphocyte therapy (ALT) (4) and tumor-infiltrating lymphocytes (TILs) (5) have been the best studied. While these approaches have not yet consistently shown great benefit for metastatic cancer (6), the conditioning chemotherapy regimen that enhances tumor responses of TIL therapy has recently been published (7).

We have conducted ex vivo cell therapy for cancer treatment using activated autologous lymphocytes, including
LAK cells, TILs and tumor-sensitized lymphocytes (8). The clinical results of trials using these activated lymphocytes, however, have demonstrated their limited tumor response in a fraction of patients with lung metastasis of renal cell carcinoma by systemic administration of effector cells (CR+PR=9%) (8). Locoregional administration of TILs has shown favorable results (77%) to reduce malignant effusions (8). These results suggest that effector cells, which express stronger and more specific cytotoxic activity, may result in better clinical efficacy, and that clinical trials of AIT using effector cells may be planned for metastatic lung tumors or locoregional administration. The success of adoptive cellular therapy depends on the ability to optimally produce cells equipped with the desired antigenic specificity, and then induce cellular proliferation while preserving the effector function and trafficking abilities of the lymphocytes (9).

In developing new approaches to AIT for patients with metastatic cancer, the increasing molecular understanding of antigen presentation and recognition has highlighted the use of professional antigen-presenting dendritic cells (DCs) (6, 9). In a previous work, we discussed a novel system for generating cytotoxic effector lymphocytes using antigenic peptides and cultured DCs, designated as peptide-pulsed DC-activated killer (PDAK) cells (10). In the present paper, we report on a clinical study of AIT using PDAK cells for patients with antigen-positive metastatic lung tumors which provides evidences in favor of the safety, feasibility and anti-tumor activity of this type of AIT.

Patients and Methods

Patients. Patients were eligible if they were HLA-A0201 or -A24 adults under 80 years old who had histologically-confirmed cancer with antigen-positive metastases that were refractory to standard therapy. Antigen expression of the tumor was ensured by serum carcinoembryonic antigen (CEA) levels, or reverse transcription-polymerase chain reaction using primers specific for Muc-1 (11), gp100 (12), Her-2 (13) and SART-3 (14). In another 2 days, the floating cells were collected as DCs. DCs were defined with typical morphology (>95% non-adherent cells, >95% CD14+, >95% CD80+, >75% CD86+, >65% CD83+ and veiled cells) and phenotype (>85% HLA class I+, >75% CD4+, >50% CD8+ and <20% CD14+) (10, 18).

Study design. The study was an open-label, non-randomized, dose-escalation study, and was performed at Hiroshima University Hospital since 2000. The protocol was approved by the institutional review board, and all of the patients gave written informed consent. The patients received either Muc-1, CEA, gp100, Her2, SART-3-PDAK cells intravenously in combination with 350,000 U IL-2 (Shionogi, Japan) weekly for 9 weeks, together with a planned dose-escalation schedule of three transfers each of 1 x 10⁷, 3 x 10⁷ and 1 x 10⁸ PDAK cells/kg for 6 patients, and with a uniform dose of 3 x 10⁷ PDAK cells/kg for the remaining 5 patients. Adverse effects and tumor responses were carefully evaluated after every three transfers. Toxicity was assessed using the National Cancer Institute common toxicity criteria version 2.0. All patients were monitored clinically using imaging analysis such as chest X-ray and computed tomographic examinations and clinical efficacies were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) (16).

DC preparation. DCs were induced using a modification of Romani et al. (17). Briefly, peripheral blood mononuclear cells (PBMCs) were collected from patients by the centrifugation of 20 ml heparinized venous blood samples on Ficoll-Conray gradients. PBMCs were allowed to adhere to culture flasks (Sumitomo Berclight, Akita, Japan) for 2 h at 37°C in RPMI-1640 medium containing 2% autologous serum. After removal of the non-adherent cells, adherent cells were cultured in RPMI-1640 medium supplemented with 2% autologous serum, 80 U/ml GM-CSF (IBL, Gunma, Japan), and 500 U/ml IL-4 (IBL). On day 5 of the culture, 100 U/ml TNF-α (IBL) was added and cells were cultured for another 2 days. The floating cells were collected as DCs. DCs were analyzed for quality assurance, and the release criteria of cultured DCs were defined with typical morphology (>95% non-adherent veiled cells) and phenotype (>85% HLA class I+, >75% HLA-DR+, >95% CD80+, >75% CD86+, >65% CD83+ and <20% CD14+) (10, 18).

Generation of PDAK cells. PDAK cells were generated as mentioned in detail elsewhere (10, 18). In brief, PBMCs were collected and fractionated into adherent and non-adherent cells. DCs, which had previously been prepared as mentioned above, were inactivated with 50 µg/ml mitomycin-C (Kyowahakko, Tokyo, Japan) and pulsed with antigenic peptide (40 µg/ml) for 2 h in RPMI-1640 medium containing 2% autologous serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The peptides used in this study are shown in Table I (19-25). The IFN-γ response of the patient’s PBMCs to the peptide was confirmed before generating the PDAK cells (26). After 4 washes of DCs, the non-adherent fraction of PBMCs (10⁷) was stimulated with peptide-pulsed DCs at a responder-to-stimulator ratio of 10, and maintained in RPMI-1640/2% autologous serum medium containing 10 U/ml IL-7 (IBL). Two days later, cells were washed and 80 U/ml IL-2 (Shionogi,

### Table I. Antigenic peptides used for PDAK cell induction.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>HLA</th>
<th>Peptide sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muc-1</td>
<td>A2</td>
<td>STAPPAHGV</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>A24</td>
<td>GVTSAPDTRPAGSTAPP</td>
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<tr>
<td>CEA</td>
<td>A2</td>
<td>YLSGADLN</td>
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<td></td>
<td>A24</td>
<td>TACFVSNL</td>
<td>22</td>
</tr>
<tr>
<td>gp-100</td>
<td>A2</td>
<td>VYFFLPDHL</td>
<td>23</td>
</tr>
<tr>
<td>Her-2</td>
<td>A2</td>
<td>KIFGSLAFL</td>
<td>24</td>
</tr>
<tr>
<td>SART-3</td>
<td>A24</td>
<td>AYIDFEMKI</td>
<td>25</td>
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After 4-h incubation at 37°C, release of 51Cr in the supernatant over the target cells at various densities in a final volume of 0.2 ml. for 1 h at 4°C with 10 Ìg/ ml of anti-TCR·', TCRV'6, 12 mAbs of PDAK cells. In some experiments, PDAK cells were incubated with 2% Triton X-100 over the peptide-pulsed target cells instead against peptide-un-pulsed target))/((maximum release) – (release by PDAK triplicate wells was calculated by the following formula: ((release

The mean percentage of the peptide/HLA-specific lysis of the triplicate wells was calculated by the following formula: ([(release by PDAK against peptide-pulsed target) – (release by PDAK against peptide-un-pulsed target)]/[(maximum release) – (spontaneous release)]) x100. The spontaneous release was obtained from the wells of peptide-pulsed target cells alone and was around 15% of the maximum release, which was obtained from wells added with 2% Triton X-100 over the peptide-pulsed target cells instead of PDAK cells. In some experiments, PDAK cells were incubated for 1 h at 4°C with 10 µg/ml of anti-TCR¿¿, TCRV¿6, 12 mAbs (Ortho Diagnostic System, Raritan, NJ, USA), then cytotoxicity assays were carried out. Killer units were the killing activity of PDAK cells multiplied by transferred cell numbers and was calculated at each transfer by the following formula: ((peptide/HLA-specific killing activity of PDAK at an E/T=20) x (transferred cell number) / (body weight x107).

\[ \text{Killer units} = \frac{\text{peptide/HLA-specific killing activity of PDAK at an E/T=20} \times \text{transferred cell number}}{\text{body weight} \times 10^7} \]

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Antibody and flow cytometry. The DCs (105) and PDAK cells (5x105) were stained with antibodies, washed, and then analyzed on FACSCan (Becton Dickinson, San Diego, CA, USA). The antibodies used were anti-class I, anti-HLA-DR, anti-CD80, anti-CD86, anti-CD-83, anti-CD14 antibodies for DCs, and anti-CD3, anti-CD4 and anti-CD8 antibodies for PDAK cells. All antibodies used were purchased from Becton Dickinson.

T-cell receptor gene usage analysis. Total RNA was extracted from 5 x 105 cells of PBMCs and PDAK cells and reverse-transcribed with random hexamer, as described previously (27). Aliquots of the cDNA were amplified by PCR in separate tubes, using V¿-specific oligonucleotides and C¿ reverse primer on a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA). The amplified DNA was confirmed by Southern blot analysis using a C¿ probe with luminol reaction. The light output detected on X-ray film was quantified using NIH-imaging software and a Macintosh personal computer.

Diagnostic single-strand conformation polymorphism. To detect the clonotype of the complementarity determining region (CDR) 3 in the PCR product of each TCR¿¿ band, the diagnostic single-strand conformational polymorphism (SSCP) technique was performed (27). In brief, 5 µl of the asymmetric PCR product, which was mixed with 5 µl of 95% formamide containing xylene cyanol and bromophenol blue, was heated at 95°C for 5 min, cooled on ice, and then loaded onto a 10% acrylamide gel. This was run at 100 V for 4 h in a cold room (4°C). The gel was then silver-stained (Silver Stain Plus, BioRad, Hercules, CA, USA).

Statistical analysis. Statistical evaluations for experimental values were analyzed using the non-parametric Student's t-test.
Results

Patients. Eleven patients with various types of cancer (6 colon, 2 pancreatic, 1 breast, 1 lung and 1 malignant melanoma) participated in the present study (Table II). There were 8 males and 3 females, and their mean age was 55, with a range from 24 to 76. Five patients had HLA-A2 haplotype and 6 had HLA-A24 haplotype. The patients’ Eastern Cooperative Oncology Group (ECOG) performance status was 0, 1, 2 and 3 in 5, 2, 2 and 2 patients, respectively. Seven patients had distant metastases in addition to lung metastases, and all but the melanoma patient had previously been treated with chemotherapy or radiotherapy, which had failed to inhibit tumor growth.

Antigen peptides used for generating PDAK cells are shown in Table III. CEA was used for patients who had high serum CEA levels. The other antigen expression was confirmed by RT-PCR analysis for biopsy samples of lung metastasis from cases 1, 5 and 8, but not done in cases 2 and 10. Muc-1 peptide was chosen for case 2 because most pancreatic cancer has been shown to express Muc-1 antigen (11), and SART-3 peptide was chosen for case 10 because SART-3 has been reported to be ubiquitously expressed (14). Before generating PDAK cells, all peptides used were confirmed in vitro to stimulate IFN-γ production from patient’s PBMCs (data not shown).

PDAK cells. PDAK cells could be generated in all patients enrolled (Table III). The total number of PDAK cells infused varied from 0.9 to 23.1x10^9 cells depending on the patients’ body weight and dose steps of the study. PDAK cells generated from 9 out of 10 patients tested showed predominant expansion of CD8 phenotype. In the dosage of step 2, PDAK cells from 7 patients expressed <50, but those from 3 patients showed ≥15 peptide/HLA-specific killer units, which mean peptide/HLA-specific activity of PDAK cells was connected with patient’s body weight and cell numbers infused. Mean values of the killer units in the step 2 dosage varied from 4 to 208 among the patients tested.

Feasibility and toxicities. Feasibility and toxicity are also shown in Table III. Dose escalation of PDAK cell transfer was performed in 6 patients, with doses of 1x10^7 and 3x10^7 PDAK cells/kg given to 6 and 5 patients, respectively, but the planned dose of 1x10^8 PDAK cells/kg could be administered to only 1 patient. The treatment was stopped in a melanoma patient with 2 transfers of gp-100-PDAK cells and in a colon cancer patient with 3 transfers of CEA-PDAK cells due to rapid disease progression. Grade 1 to 3 toxicities, including an increase of transaminase, headache and fever, were found in the breast cancer patient and 2 colon cancer patients who were treated with CEA-PDAK cells. No correlation was observed between toxicities and total numbers of PDAK cells transferred.

Tumor response. Tumor response is shown in Table III. When focused on lung metastasis, growth arrest of the lung tumor, including PR or SD responses, was observed in 7
out of 11 patients, although 4 of the 7 patients showed tumor progression in distant metastases other than the lung. A pancreatic cancer patient (case 1, Figure 1), who received $3 \times 10^7$ Muc-1-PDAK cells/kg, showed <30% tumor reduction in the lung; in this patient, other distant metastases to the pleuro-peritoneum and lymph nodes were stable for a period of 4 months. A decrease of serum CEA levels was observed in 3 of the 7 colorectal cancer patients, one of whom (case 6, Figure 2) showed growth arrest of lung metastasis with $23.1 \times 10^9$ CEA-PDAK cells, but showed no other tumor response in the pleura or lymph nodes. In an overall assessment, 1 PR (a pancreatic cancer patient), 3 SDs (1 breast and 2 colorectal cancer patients) and 6 PDs were observed.

There was no relationship between tumor response and total numbers of PDAK cells infused. Regarding the killer units, 7 patients with PR or SD response at lung lesions had mean value ± standard deviation of 87±54 activity, while 3 PD patients had that of only 11±6 activity, in the dose level of step 2 (Figure 3). There was a significant difference between these values ($p<0.05$).

**T-cell receptor analysis.** A 52-year-old male patient with pancreatic cancer (case 1) showed a partial response in lung metastasis by Muc-1-PDAK cell transfer (Figure 1A, B). The PBMCs of the patient demonstrated a diverse expression of TCRVβ gene usage before stimulation, while the transferred Muc-1-PDAK cells showed preferential usage of TCRVβ3, 6, 13.1, 13.2, 14 and 17 (Figure 1C).

A 68-year-old male patient with colon cancer (case 6) showed growth arrest of lung metastasis with a decrease in serum CEA level due to CEA-PDAK cell transfer (Figure 2A, B). TCRVβ gene usage analysis clearly demonstrated a difference in TCRVβ expression before and after stimulation, and the preferential usage of TCRVβ1, 2, 3, 5.2, and especially of TCRVβ6 and 12 was indicated (Figure 2C). The CEA-PDAK cells killed T2 target cells pulsed with the CEA peptide, whose cytotoxicity was significantly abrogated in the presence of anti-TCRVβ12 antibody ($p<0.05$), but this did not occur in the presence of anti-TCRVβ6 antibody or the irrelevant control antibody (Figure 2D). SSCP analysis showed clonotopic band pairs of the TCRVβ12 (Figure 2E).
In the present study, we demonstrated the safety of AIT using PDAK cells for metastatic lung tumors. This is to be expected since AIT using activated lymphocytes has been shown to be essentially safe except in combined administration of high dose IL-2 (28, 29). We also showed that our method of AIT using PDAK cells was feasible at an administration of $3 \times 10^7$ cells/kg, but not at a dose of $1 \times 10^8$ PDAK cells/kg. Importantly, one partial response was observed in a colon cancer patient treated with CEA-PDAK cells. A 68-year-old male patient with colon cancer who had lung, pleural and mediastinal lymph node metastases (case 6) was administered intravenously with CEA-PDAK cells, and CT scan examination was performed prior to (A) and after (B) the treatment. Serum CEA levels decreased from 40.5 to 21.8 ng/ml by the treatment. T-cell receptor gene analysis for CEA-PDAK cells was performed prior to and after the stimulation (C). The cytotoxicity of CEA-PDAK cells was determined against CEA peptide-pulsed T2 cells at an E/T ratio of 25 in the presence of the antibodies indicated (D). SSCP analysis was performed for detecting clonotypes of TVR/Vβ12 (E).
pancreatic cancer patient with a dose of $3 \times 10^7$ Muc-1-PDAK cells/kg. Although Simon et al. state that dose-finding studies may not be necessary in cell therapy such as tumor vaccine trials (30), our results suggest that the dose of $3 \times 10^7$ PDAK cells/kg is the limitation of our culture system and may be the least dose required for tumor responses.

We could observe only one PR and 6 SDs in metastatic lung lesions and no response in other lesions including liver, lymph node and effusion, by the intravenous systemic administration of PDAK cells. It has been reported that the trafficking of effector cells toward the tumor site is critical for tumor response (5-9). For example, other researchers have attempted to address hepatic tumors by arterial infusion of effector cells through the hepatic artery (31). By intravenous administration, 100% effector cells can reach lung lesions. Moreover, PDAK cells have been shown to express chemotactic chemokine receptor 5 (18), which is reported to be required for locoregional trafficking of effector lymphocytes (32). However, these may not explain the unsatisfactory results of lung lesions in this study by PDAK cell transfer. To augment the efficacies, we may pay more attention not only to generating the effector cells of high quality, but also to conditioning the host immune regulation systems before AIT using PDAK cells. Dudley et al. (7) reported the remarkable enhancement of the clinical efficacy of AIT against malignant melanoma using TILs by pre-treating patients with non-myeloablative lympho-depleting chemotherapy.

We measured the peptide/HLA-specific killing activity of PDAK cells and calculated the killer units upon transfer; the number of killer units indicates the total of peptide/HLA-specific killing activity of transferred PDAK cells. It is an interesting question whether or not this parameter of killing activity correlates with clinical responses to AIT using PDAK cells. In the present trial, the number of killer units in PR and SD patients was significantly higher than that in PD patients. In previous AIT trials using LAK cells, it has been reported that neither tumor reduction nor clinical toxicity correlates with dose or with the cytolytic activity of LAK cells, nor are there any correlations with other laboratory parameters including base-line lymphocyte count and IL-2-induced lymphocytosis (29). However, Kawakami et al. (23) have reported that tumor regression is correlated with the recognition of gp100 epitopes by the adoptively administered TILs in treating patients with melanoma. Like TILs, but unlike LAK cells, PDAK cells have been shown to recognize tumor cells in a peptide/HLA-specific manner (10, 18). Therefore, the peptide/HLA-specific killing activity of PDAK cells may be involved in clinical results of tumor responses. In addition,
measurement of peptide/HLA-specific killing activity is also important for the quality control of PDAK cells used in the trials (30). This issue for the quality control of the effector cells remains to be addressed in future clinical trials.

In order to generate PDAK cells of high quality, the selection of appropriate peptides may be critical. We have previously reported that the CEA peptide, which can stimulate CTL precursors to produce IFN-γ, differs in individuals among HLA-A24 healthy donors and colorectal cancer patients when tested with a whole blood assay using a CEA peptide panel (26), although CEA652 has been shown to have the most potent binding affinity for HLA-A24 molecules and to induce CEA-reactive CTLs (22). Kedl et al. have shown that PDAK cells used in the measurement of peptide/HLA-specific killing activity is also important for the quality control of PDAK cells used in the trials (30). This issue for the quality control of the effector cells remains to be addressed in future clinical trials.

In conclusion, AIT for antigen-positive metastatic lung tumors using PDAK cells was found to be both safe and feasible. Based on the present data on dosages, tumor response should be examined in a future clinical trial. Large-scale clinical trials are on-going to prove the efficacy of AIT using PDAK cells against metastatic lung tumors.

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References


