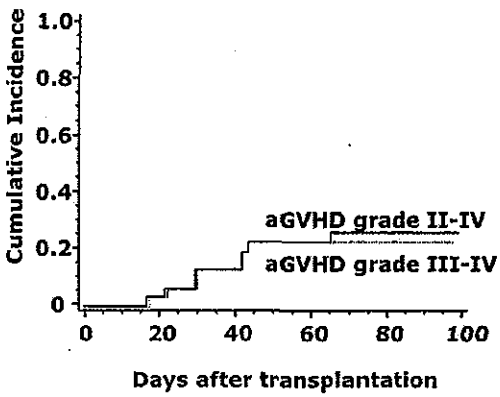

Fig. 3 Development of acute graft-*versus*-host disease (GVHD). Grade II-IV and III-IV acute GVHD developed in 27% (95% confidence interval, 11–43%) and 23% (95% confidence interval, 7.4–39%) of the patients, respectively. Median onsets of grade II-IV and III-IV acute GVHD were day 36 (range, day 17–66) and day 30 (range, day17–44), respectively.

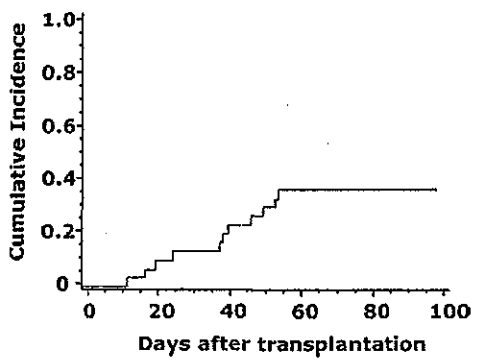

Fig. 4 Development of cytomegalovirus reactivation. Reactivation of cytomegalovirus was documented in 11 patients (37%) on a median of day 40 (range, day 13–55).

corticosteroids at the onset of infections. Reactivation of CMV was documented in 11 patients (37%) on a median of day 40 (range, day 13–55; Fig. 4). Eight of them had been treated with corticosteroids at the onset of CMV antigenemia. None of them developed CMV-related diseases. One patient developed hemorrhagic cystitis with adenovirus and BK virus infection.

Pre-Engraftment Noninfectious Fever. Seven patients with documented infection before engraftment were excluded from the analysis of pre-engraftment reaction (Table 4). Eighteen patients developed noninfectious fever before neutrophil engraftment (Fig. 5). Noninfectious high-grade fever often coexisted with eruption, diarrhea, and weight gain, starting on a median of day 9. Pathological examination of eruption from 8 patients revealed nonspecific inflammatory reactions and was not compatible with GVHD.

Survival. As of January 2004, a total of 11 patients remained alive. Median follow-up of the survivors and all of the enrolled patients were 238 days (range, 169–485) and 125 days (range, 26–485), respectively. Primary diseases recurred in 3 patients. Estimated 1-year OS and EFS were 32.7% (95% CI, 14.3–51.1%; Fig. 6) and 22.2% (95% CI, 5.9–38.5%; Fig. 7), respectively. Neither cell dose nor HLA disparity was associated with OS (Table 2).

DISCUSSION

Because CB contains a small amount of hematopoietic stem cells and stem cell boost or donor lymphocyte infusion is not available after UCBT, graft failure has been a major concern in adult UCBT. The present study demonstrated the feasibility of RI-UCBT for adult patients, in addition to pediatric patients (21). In this study, 26 of the 30 patients (87%) achieved durable engraftment, and 28 patients achieved complete donor chimerism by day 60, including 2 patients who died before engraftment. Interestingly, 4 patients with severe aplastic anemia, which has been associated with a high incidence of graft rejection (22), achieved complete chimerism after our reduced-intensity regimen. These findings suggest that the combination of fludarabine, melphalan, and low-dose TBI might be more immunosuppressive than conventional myeloablative regimens, creating niche for CB to engraft. Alternatively, CB may exert a strong graft-*versus*-host effect, making room for stable engraftment of stem cells.

Delayed hematopoietic recovery and infection during neutropenia are the significant concerns in adult UCBT. Laughlin *et*

Table 4 Characteristics of pre-engraftment reaction (n = 23)

Temperature	
38.0–38.9°C	2
39.0–39.9°C	10
≥40.0°C	7
Day of peak body temperature	9 (5–12)
Serum levels of CRP[a] (mg/dl)	13.8 (0.5–18.9)
Day of peak serum levels of CRP	10 (8–16)
Diarrhea	11
Eruption	10
Jaundice	5
Use of corticosteroid	13
Good response to corticosteroid	7

[a] CRP, C-reactive protein.

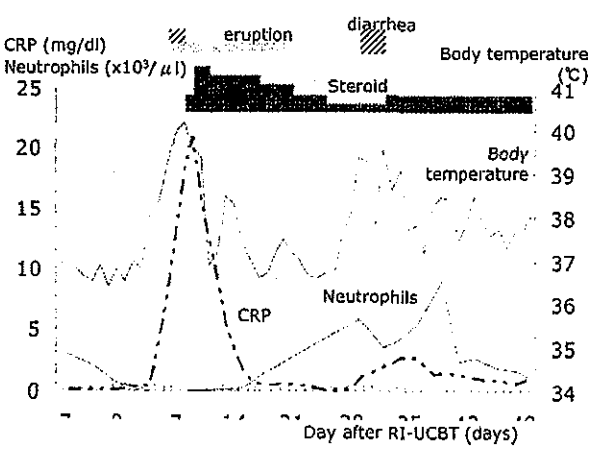

Fig. 5 Clinical course of a patient who developed pre-engraftment fever. Immune-reactions display two peaks, at around day 9 and day 18.

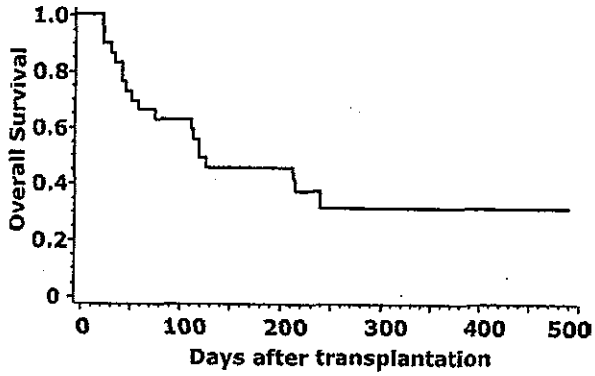

Fig. 6 Probability of overall survival after reduced-intensity unrelated cord-blood transplantation. Estimated 1-year overall survival was 32.7% (95% confidence interval, 14.3–51.1%).

al. (23) reported neutrophil recovery in 90% of patients by a median of 27 days after UCBT, which was significantly delayed compared with allo-HSCT. The delay has been attributed to the limited cell dose in the reports on myeloablative UCBT. The median nucleated cell dose in our study (3.1×10^7/kg) was greater than those in some reports from Western countries (2.1×10^7/kg; Ref. 9). The low median body weight (52 kg) in the Japanese population may favor neutrophil engraftment, whereas our results showed no association between the cell dose and engraftment in the small sample size. In the present study, median time to engraftment was 17.5 days (range, 10–54 days), which was much faster than that reported in previous studies on myeloablative UCBT (7–9). Our results were comparable with the report on adult RI-UCBT by Barker *et al.* (21). Their results showed neutrophil engraftment on a median of 26 days after busulfan/fludarabine/TBI 2 Gy and 9.5 days after cyclophosphamide/fludarabine/TBI 2 Gy. Whereas the reason for the difference remains unclear, these findings suggest that fludarabine-based reduced-intensity regimens enable rapid and stable engraftment.

TRM within 100 days was 27% in this study, which is lower than those reported on myeloablative UCBT (Refs. 7, 9, 24; 32–51% in pediatric patients and 56–63% in adults). Given the relatively old age (median, 58.5 years) and advanced stages of the primary diseases, our reduced-intensity preparative regimen probably decreased TRM. Our TRM within 100 days is comparable with that of 28% in adult RI-UCBT by Barker *et al* (21).

All of the patients tolerated our preparative regimen without grade IV RRT (Bearman's criteria; Ref. 2). Four patients developed grade III RRT with common involvements of the gut, kidney, and liver (Table 3). We used melphalan, which has dose-limiting toxicities of the gut and liver (25). These remained mild without hepatic veno-occlusive disease. Because renal toxicities of fludarabine, busulfan, and TBI 4 Gy are reportedly minimal, the high incidence of renal toxicity might be attributable to concomitant administration of nephrotoxic agents such as cyclosporin and antibiotics. Elderly patients might be susceptible to RRT. We plan to investigate optimal dosages of cyclosporin in RIST for elderly patients. Because TBI, even at a low dose, sometimes causes significant late toxicities in the lung (22), long-term follow-up is required.

Little information on GVHD after RI-UCBT is available. In the present study, the incidences of grade II-IV and III-IV acute GVHD and chronic GVHD were 27%, 23%, and 23%, respectively, whereas some reported those to be 33–44%, 11–22%, and 0–25%, respectively, in myeloablative UCBT (7, 8, 26). There are no significant differences in the incidences of GVHD between myeloablative UCBT and RI-UCBT. This is similar to the GVHD incidences in myeloablative allo-HSCT and RIST (27). Median onset of acute GVHD was 36 days (range, 17–66 days) in the present study, which was comparable with that of myeloablative UCBT (7, 8, 26). In contrast, the achievement of complete donor chimerism and the onset of acute GVHD are delayed in RIST compared with myeloablative allo-HSCT (27, 28). CB might have a potential of intense graft-*versus*-host effect, allowing niche for early engraftment. The characteristics of GVHD after RI-UCBT remain to be investigated, including different organ involvements and response to immunosuppressive treatment.

Interestingly, 20 patients developed inflammatory reactions before engraftment (Table 4). These reactions included noninfectious high-grade fever, eruption, diarrhea, and jaundice, starting on a median of day 9. Because the reactions preceded engraftment (median, day 17.5), we speculated that some form of immune reaction that is not categorized as acute GVHD occurs after RI-UCBT without achieving engraftment. The pre-engraftment fever has been reported on rare occasions in previous reports of UCBT and might be similar to those observed after haploidentical transplantations. Antithymocyte globulin and corticosteroids, which have strong immunosuppressive properties, were commonly used in previous studies on UCBT (9), whereas neither was used in the present study. Immunosuppressive treatment with corticosteroids was effective for the pre-engraftment fever. These findings support that immune-mediated reactions after UCBT might manifest easily with the present regimen. The doubling time of cultured CB $CD34^+$ cells is 7–10 days, which is several hundred-fold faster than that of cultured adult marrow cells (29). Mononuclear cells from CB display a unique cytokine profile such as comparable levels of

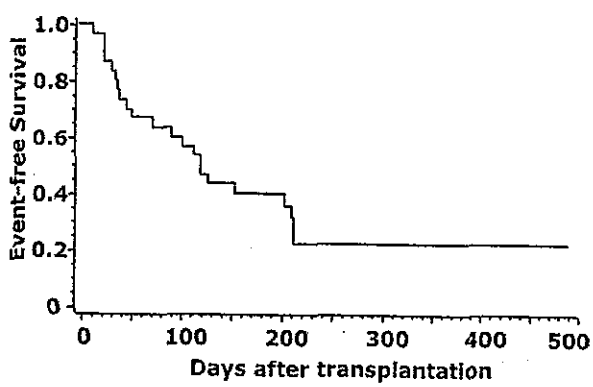

Fig. 7 Probability of event-free survival after reduced-intensity unrelated cord-blood transplantation. Estimated 1-year event-free survival was 22.2% (95% confidence interval, 5.9–38.5%).

interleukin (IL) 2, IL-6, and tumor necrosis factor α, reduced levels of IFN-γ and IL-10, and complete absence of IL-4 and IL-5 (30, 31). Pre-engraftment fever is possibly attributable to a cytokine storm induced by massive proliferation of cells with a unique cytokine profile. Another possibility is homeostasis-driven proliferation of naive T cells in highly immunosuppressed individuals, as demonstrated in murine models (32, 33). This reaction is reportedly associated with cytotoxic cytokines (32, 33). Fever as a transient response to contamination with maternal blood or cells during CB collection cannot be excluded (34). Reactivation of human herpesvirus 6 might be associated with this complication (35). If pre-engraftment fever exerts some antitumor effects, it is reasonable that patients with advanced and chemorefractory hematological diseases achieved long-term remission after RI-UCBT in the present study.

Infection is a common and significant problem in myeloablative UCBT (8, 9, 24), but little is known in RI-UCBT. The present study demonstrated that infection is also problematic in RI-UCBT. Twelve patients developed infection in this study, 9 of whom had been on corticosteroid therapy. Eight of 11 patients with CMV antigenemia had received corticosteroids. Delayed immunological reconstitution with or without GVHD, pre-engraftment fever, and corticosteroids may be risk factors for infection. Appropriate management of GVHD and pre-engraftment fever warrants additional investigation.

One-year OS was 35% in the present study, showing that some patients with advanced hematological malignancies can achieve durable remission after RI-UCBT. Contrary to our prediction, primary diseases recurred only in 3 patients. The candidates for RI-UCBT have extremely poor prognosis with conventional salvage chemotherapy. These findings suggest that RI-UCBT exerts strong antitumor activity and is promising for patients with refractory hematological malignancies without an HLA-identical sibling or an unrelated donor. In contrast, it is premature to apply RI-UCBT to low-risk diseases.

In conclusion, our study demonstrated the feasibility of RI-UCBT for adult patients with advanced hematological diseases, although the limitations included the small sample size and short follow-up. If CB is feasible for adults as an alternative stem cell source, RI-UCBT may become the choice of treatment for patients with advanced hematological diseases that are incurable with conventional treatments. RI-UCBT is particularly appealing for patients who require urgent treatments. Although RI-UCBT is currently associated with a high TRM, this study provided a rationale for continuing our clinical trials. Additional investigations need to focus on minimizing adverse effects including RRT, GVHD, and pre-engraftment immune reactions, whereas preserving graft-*versus*-leukemia effects.

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Reduced-Intensity Allogeneic Hematopoietic Stem-Cell Transplantation as an Immunotherapy for Metastatic Colorectal Cancer

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Background. Allogeneic stem-cell transplantation (allo-SCT) can induce curative graft-versus-leukemia reactions for hematologic malignancies through allogeneic immunity. Because the gastrointestinal tract is a target of graft-versus-host disease (GvHD), colorectal cancer might be a candidate for allo-SCT.

Methods. Four patients with metastatic colorectal cancer underwent reduced-intensity stem-cell transplantation (RIST) in the National Cancer Center Hospital between July 2002 and February 2003. Three patients received transplants from an human leukocyte antigen (HLA)-identical related donor, and the remaining patient received selected CD34-positive cells from a two-loci HLA-mismatched donor. The basis of preparative regimen was busulfan 4 mg/kg for 2 days and fludarabine 25 mg/kg for 6 days.

Results. All the patients tolerated the preparative regimen and achieved engraftment without significant toxicities. All developed acute or chronic GvHD. Although serum levels of CA19–9 and carcinoembryonic antigen were transiently elevated after RIST in all the patients, the levels subsequently decreased below the levels from before RIST in all but one patient. Three had measurable lesions before RIST, one achieved partial response, and the others stable disease, which was durable for 120 and 60 days. Three patients died; the causes of death were progressive disease, GvHD, and accident. Postmortem examination was obtained for two patients; in one patient, the peritoneal metastatic lesions macroscopically disappeared, and in the other patient, the supraclavicular lymph node disappeared while the other measurable lesions remained stable.

Conclusions. All the patients showed some evidence suggesting the presence of a graft-versus-tumor effect for colorectal cancer, which should be confirmed in a future prospective trial.

Keywords: Graft-versus-tumor effect, Graft-versus-host disease, Allogeneic immunity, Fludarabine, Carcinoembryonic antigen.

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A new strategy of allogeneic hematopoietic stem-cell transplantation (allo-SCT) using a reduced-intensity preparative regimen (reduced-intensity stem-cell transplantation [RIST]) was developed to decrease regimen-related toxicity (RRT) while preserving an adequate antitumor effect (*1, 2*). Different pioneering conditioning regimens for RIST have been investigated: those including purine analogs (*1–3*) and total body irradiation (TBI) combined with potent immunosuppressants (*4*). Because clinical studies on RIST have focused in hematologic malignancies, limited information is available on solid tumors (*5*), including renal cell carcinoma (RCC) (*6*), breast (*7, 8*), lung (*9*), ovarian (*10*), and colon cancer (*11*).

Because the epithelium is the target of graft-versus-host disease (GvHD), any types of carcinoma arising from the epithelial tissues such as keratinocytes, fibroblasts, exocrine glands, hepatobiliary trees, and gastrointestinal tract are theoretically susceptible to a graft-versus-tumor (GvT) effect. Murine models have provided some evidence for an allogeneic immune-mediated antisolid-tumor effect (*12*). Porter et al. (*13*) conducted a phase I clinical trial to determine whether a GvT effect could be observed after primary donor lymphocyte infusion (DLI) without stem-cell support in patients with primary cancers. Three of the four patients with acute GvHD and late chimerism responded to DLI. Eibl et al. (*14*) demonstrated that allogeneic T cells collected during GvHD could mediate a cytotoxic effect against breast cancer cell lines. Childs et al. (*15*) reported the results of 19 patients who underwent RIST for metastatic RCC. Seven patients achieved complete response and seven partial response (PR). The tumor response was associated with the development of GvHD. These results suggest that a GvT effect does exist in RIST for a variety of solid tumors, although long-term prognosis remains unknown.

Colorectal cancer is the second cause of cancer death (*16*), and prognosis for patients with unresectable and refractory to chemotherapy metastasis is poor. Development of novel therapeutic strategy is required. Because the gastrointestinal tract is the common target of GvHD, colorectal cancer might be sensitive to allogeneic immunity. We report four

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patients who underwent RIST for metastatic colorectal cancer.

PATIENTS AND METHODS

Patients

Between July 2002 and February 2003, four patients with colorectal cancer underwent RIST. All the patients had progressive metastatic disease, which was refractory to any conventional anticancer therapies including fluorouracil and irinotecan. Peripheral blood stem-cells were mobilized by subcutaneous injection of granulocyte colony-stimulating factor, 10 μg/kg, and harvested with the target cell dose of greater than 2.0×10^6/kg CD34+ cells.

The RIST program was approved by the Institutional Review Board of the National Cancer Center Hospital. A written informed consent was obtained from all the patients and donors.

Conditioning and Donors

The basis of preparative regimen was busulfan 4 mg/kg for 2 days and fludarabine 30 mg/kg for 6 days. Patient 1 received additional TBI 4.0 Gy in two fractions. Because a human leukocyte antigen (HLA)-identical donor was not available for patient 1, he underwent RIST from his two-loci mismatched brother-in-law with CD34+ cell selection. The number of simultaneously infused CD3+ cells was 2.5×10^4/kg. The remaining three patients had HLA-identical related donors: a sibling (patient 2 and 4) and an offspring (patient 3). Patient 2 received additional rabbit antithymocyte globulin (ATG) 2.5 mg/kg for 2 days to ensure durable engraftment. Since October 2002, ATG was omitted from the preparative regimen in the protocol of RIST for solid tumors, and ATG was not given to patient 3 and 4.

Engraftment and Management of GvHD

Recipient-donor chimerism in peripheral blood mononuclear cells (PBMC), T cells, and granulocytes were analyzed monthly after transplantation using polymerase chain reaction of informative short tandem repeat (*17*). GvHD prophylaxis was intravenous cyclosporine 3 mg/kg or oral cyclosporine 6 mg/kg from day −1. Because the incidence of acute GvHD is approximately 10% in RIST from an HLA-matched sibling after an ATG-containing regimen in our institution (*18*), we tapered cyclosporine early and rapidly over a 2-week period to enhance a GvT effect. The diagnosis of GvHD was made in concert with biopsy of the skin or the gastrointestinal tract. Acute and chronic GvHD were graded according to the consensus criteria (*19, 20*). Grade II to IV acute GvHD was treated with 2 mg/kg per day of methylprednisolone in addition to cyclosporine.

Supportive Measures

All the patients stayed in reverse isolation in a laminar airflow-equipped room and received prophylaxis with trimethoprim/sulfamethoxazole or pentamidine inhaler and ciprofloxacin against *Pneumocystis carinii* and bacterial infection, respectively. Fluconazole was administered for antifungal prophylaxis with a dose ranging from 200 to 400 mg/day. Herpes virus prophylaxis with acyclovir was also given as previously described (*21*). Cytomegalovirus pp65 antigenemia was routinely monitored once a week. When antigenemia was detected, preemptive therapy with ganciclovir was initiated as previously reported (*22*).

Evaluation of Tumor Response

All patients underwent computed tomography (CT) scanning before RIST and monthly after RIST to evaluate the tumor response. Serum levels of CA19–9 and carcinoembryonic antigen (CEA) were monitored weekly using chemiluminescent enzyme immunoassays (Lumipulse CA19–9-N, and Lumipulse CEA-N, Fujirevio, Tokyo, Japan, respectively).

Three patients (patient 2, 3, and 4) had measurable lesions before RIST, and tumor response was defined according to the response evaluation criteria in solid tumor (RECIST) (*23*) in these patients. Postmortem examination was available in the other two patients (patient 1 and 4).

RESULTS

Engraftment, Regimen-Related Toxicities, and GvHD

All the patients tolerated the preparative regimens with minimum RRTs. The neutrophil count reached 0.5×10^9/L on day 10 in patients 1, 3, and 4 and on day 12 in PATIENT 2. On day 30, chimerism analysis in PBMC showed full donor-type chimerism in all the patients. Grade II to IV acute GvHD developed in 3 patients, on median of 23 (20–47) days after RIST. Patient 1 developed grade IV acute GvHD after DLI to induce a GvT effect. The patient died of multiorgan failure caused by acute GvHD on day 62. The signs of GvHD in the remaining two patients spontaneously disappeared without additional immunosuppressant. Among two patients who survived over 100 days, both had chronic extensive GvHD.

Tumor Responses and Outcomes

Tumor responses were shown in Table 1. Although serum levels of CA19–9 and CEA transiently elevated after RIST in all the patients, the levels subsequently decreased below the levels before RIST in all but one patient (patient 4). Three patients had measurable lesions before RIST; one (patient 3) achieved PR, one (patient 2) stable disease (SD), which was durable for 120 days, and one (patient 4) progressive disease (PD).

Three patients died; the causes of death were GvHD (patient 1), PD (patient 2), and accident (patient 4). Postmortem examination was obtained for two patients; in patient 1, the peritoneal metastatic lesions macroscopically disappeared while microscopically detectable lesions remained. In patient 4, the supraclavicular lymph node metastasis disappeared while the other measurable lesions remained stable.

CASE PRESENTATION

Patient 1

A 44-year-old man underwent RIST from his two-loci mismatched brother-in-law in July 2002 for the treatment of metastatic rectal cancer. He had peritoneal metastasis, and no measurable lesions were documented before RIST. After preparative regimen consisting of fludarabine, busulfan, and 4 Gy TBI, he received CD34-positive stem cells.

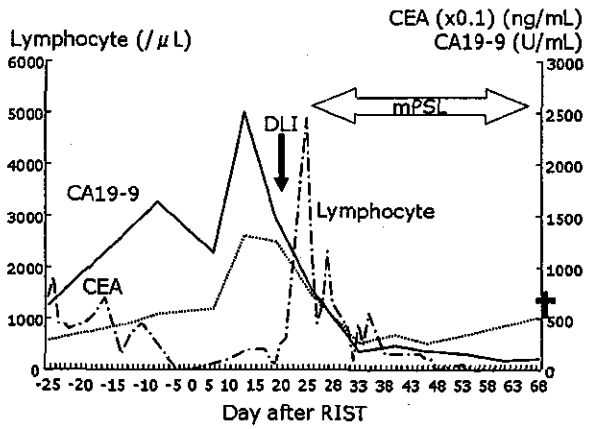

FIGURE 1. Clinical courses of patient 1. After engraftment and donor lymphocyte infusion (DLI), serum levels of tumor markers declined, and necropsy revealed that residual tumors in the peritonea shrunk. CEA, carcinoembryonic antigen; methylprednisolone, mPSL; RIST, reduced-intensity stem-cell transplantation.

His clinical courses was uneventful until day 21, when we added 4.0×10^6/kg CD3+ cells to improve immune recovery. The patient developed maculopapular rash, watery diarrhea, and jaundice on day 23. Based on the histopathologic examination of the skin, a diagnosis of grade IV acute GvHD was made. We initiated steroid-pulse therapy, but his condition deteriorated rapidly. He finally died of multiorgan failure caused by acute GvHD on day 62. Postmortem examination showed that the metastatic lesions in the peritoneum disappeared, whereas residual adenocarcinoma cells were observed by histopathologic examination.

Serum levels of CEA and CA19–9 increased from 45.7 ng/mL and 1388 U/mL before transplant to 131.0 ng/mL and 2507 U/mL on day 15, respectively. After DLI, serum levels of both values decreased rapidly (Fig. 1).

Patient 2

A 52-year-old woman underwent RIST from an HLA-identical sibling in August 2002 for metastatic rectal cancer. The metastatic lesions involved the liver and the both lungs. The liver lesions were measurable targets (Fig. 2). Because she had allergic reaction to the contrast agents on day 30, she received CT after day 60 without contrast agents. The patient had not developed acute GvHD, and we tapered off cyclosporine from day 35 until day 49 to enhance a GvT effect. Her clinical courses were uneventful until day 90, when she developed mucositis caused by chronic GvHD. To further augment the GvT effect, we withheld any immunosuppressive agents. The oral lesions had subsided spontaneously until day 120. Sequential abdominal CT scans failed to show progression until day 120 (Fig. 2). However, repeated CT scan on day 150 revealed an extensive progression of the metastatic lesions in the liver and the pleura (Fig. 2). She died of disease progression on day 172. An autopsy was denied by her family. The best and final responses were SD and PD, respectively. The duration of SD was 120 days.

Serum levels of CEA and CA19–9 were increased with transient decrease after engraftment until chronic GvHD developed and continued to decrease while the oral lesions persisted. Their serum levels were inversely associated with the severity of chronic GvHD (Fig. 3).

Patient 3

A 59-year-old woman underwent RIST from her HLA-identical offspring in October 2002. She presented metastatic

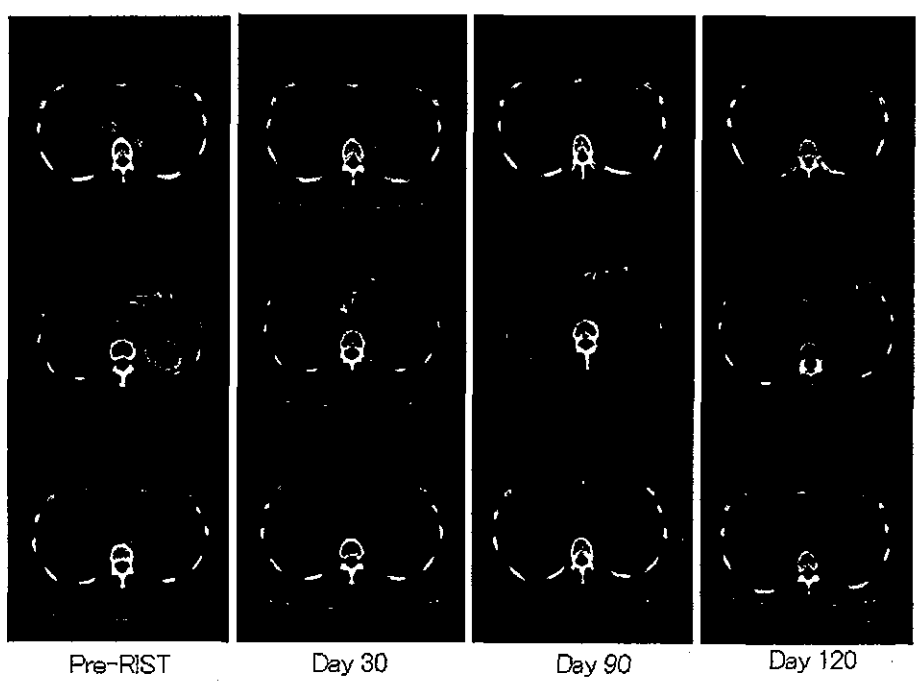

FIGURE 2. Changes of the metastatic lesions of the liver of patient 2. The metastatic lesions in the lung remained stable till day 120. However, metastatic lesions expanded with carcinogenic pleuritis.

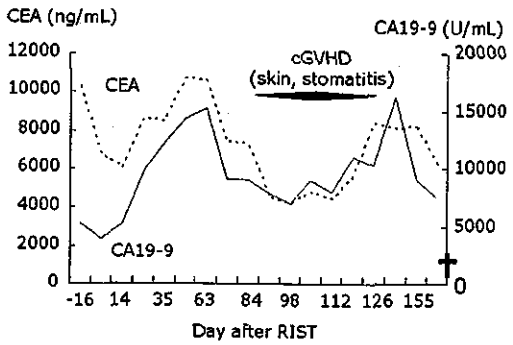

FIGURE 3. Clinical courses of patient 2. Serum levels of CEA and CA19–9 were inversely associated with the severity of chronic graft-versus-host disease (cGvHD).

lesions in the liver and the lungs. The pulmonary lesions were defined as measurable targets. After engraftment on day 10, she developed maculopapular rash on the trunk on day 20, which was histopathologically diagnosed as grade II acute GvHD. Mild but significant disease progression was detected on the follow-up CT scan on day 30 (Fig. 4). Because the cutaneous GvHD had resolved spontaneously by day 45, we started tapering of cyclosporine from day 50. The follow-up CT scans of the chest on day 60 showed a significant decrease in the size of target lesions. Thereafter, lung metastatic lesions progressed gradually again after the disappearance of acute GvHD, and donor lymphocyte containing 6.4×10^7/kg CD3+ cells were infused on day 181. She developed extensive chronic GvHD involving the skin and the lung, and size of the metastatic lesions decreased again. As of March 2004 (17 months after RIST), the best and final responses are both PR. Serum levels of CEA and CA19–9 were inversely associated with the development of GvHD (Fig. 5).

Patient 4

A 52-year-old woman with advanced colon cancer received RIST from an HLA-identical sibling in January 2003. Her multiple metastatic lesions included the liver, the lung, the peritoneal, multiple lymph nodes, and the residual colon. Liver metastases and lymph nodes were defined as measurable lesions. New metastatic lesions appeared in the lung on day 60, and the best response was PD. We tapered cyclosporine rapidly to enhance a GvT effect. With the development of grade II skin GvHD on day 38, the metastatic lesion of the supraclavicular lymph node decreased in size, and serum levels of CEA and CA19–9 decreased (Table 1). She was stable until day 88, when she accidentally fell on the floor. She suffered from fatal head injury. An autopsy revealed complete disappearance of the metastatic lesion in the supraclavicular lymph node. The other lesions were stable in size. The final response was PD.

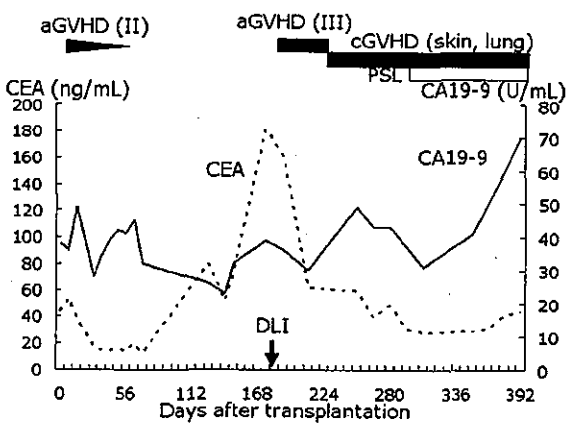

FIGURE 4. Evaluation of metastatic lesions in the lung of patient 3. Repeated computed tomography scans of the chest showed that metastatic lesions shrunk after the development of GvHD.

DISCUSSION

Allo-SCT has a considerable risk of transplant-related mortality. Development of optimal preparative regimens for colorectal cancer is an important issue for future clinical trials. With RIST procedure, all of the four patients achieved durable engraftment within 30 days of transplant. These findings suggest that our reduced-intensity regimen is sufficient to assure engraftment in RIST for metastatic colorectal cancer. Because most patients with advanced colorectal cancer are heavily treated with chemotherapeutic agents such as fluorouracil and irinotecan (*24*), the risk of graft rejection will be low compared with other solid tumors, which are rarely treated by cytotoxic agents.

RRT should be critically evaluated in developing allo-SCT. All of our patients had been treated heavily by surgery or cytotoxic chemotherapy before RIST. However, we demonstrated that RRT was mild in all the organs and that all the patients tolerated the procedure well. Grade 3 to 4 toxicity according to the Bearman's criteria (*25*) were not observed in any patients. We omitted the use of methotrexate to enhance a GvT effect. Because methotrexate causes mucosal damages, this might have contributed to the amelioration of gastrointestinal damages in this study. Our experience indicates that fludarabine/busulfan-based preparative regimens are safe and tolerable for patients with advanced colorectal cancer.

Concerning the efficacy of RIST for colorectal cancer, all the patients showed some evidence of antitumor effects. Serum levels of CEA and CA19–9 decreased significantly, either after engraftment (patient 1) or development of GvHD (patient 2 and 3). There might be a debate as to whether serum levels of CEA and CA19–9 can be reliable surrogate markers of treatment response (*26*). However, one patient (patient 3) achieved durable PR (*23*). In another patient (patient 1), postmortem examination showed marked reduction of the tumor size. Although patient 4 had supraclavicular lymph node, this lesion disappeared after development of acute GvHD. Although different conditioning regimens and GvHD prophylaxis methods were used in this study, these findings indicate that allo-SCT is promising for metastatic colorectal cancer.

We have demonstrated the feasibility of allo-SCT for colorectal cancer; however, there are some problems to be discussed. First, the precise mechanism of the GvT effect on solid tumors including colorectal cancer remains unknown. Disease regression associated with cyclosporine withdrawal, complete donor chimerism, and GvHD provides evidence

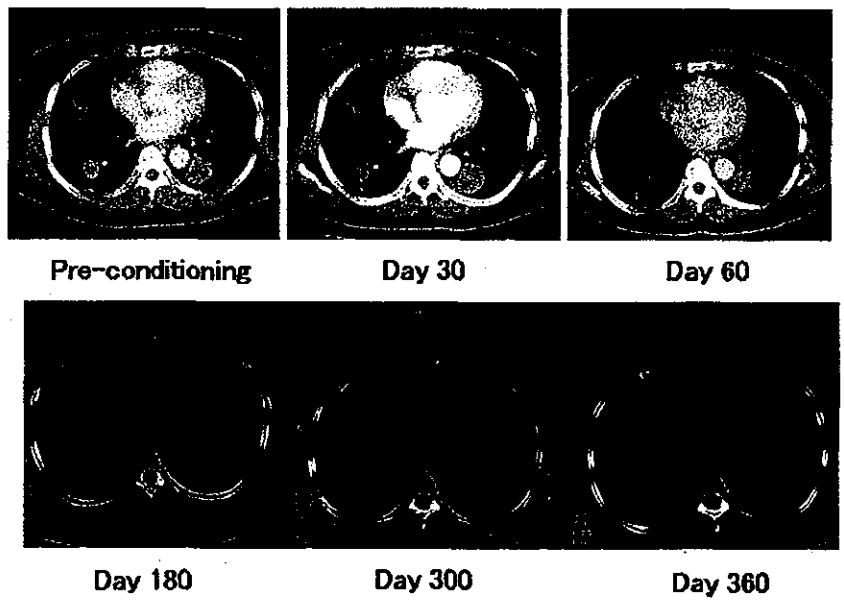

FIGURE 5. Clinical courses of patient 3. Serum levels of CEA and CA19–9 decreased after the development of GvHD.

that cytotoxic donor T cells play an important role in GvT effects. Recent studies on RIST for RCC have suggested that distinct T-cell populations recognizing tumor-specific antigens or minor histocompatibility antigens are involved in the GvT effect (*27–29*). Despite our attempt to detect anti–CEA-specific cytotoxic T lymphocyte by enzyme-linked immunosorbent spot assay, the laboratory system using HLA–A24-restricted peptides has not been established. Our effort to establish the system continues. Cloning of cytotoxic T cells and identification of target minor antigens are essential in the development of RIST for colorectal cancer. On the other hand, some investigators suggested that the local cytokine storm associated with the early phase of allogeneic transplantation plays an important role in GvHD (*30*). Furthermore, it is interesting that tumor markers declined promptly after engraftment in patient 1, suggesting the existence of an anti-colorectal cancer mechanism other than the GvT effects associated with GvHD. Allo-SCT using CD34-positive cell selection lowered the possibilities of subclinical GvHD immediately after engraftment. The GvT effect is thus unlikely to be associated with GvHD. The simultaneous decline in CEA might be attributable to a cytokine storm or immune dysregulation associated with engraftment.

Second, we should identify what types of cancer respond to alloimmunity. RCC, which is believed to be immunogenic, is promising in allogeneic immunotherapy (*15*). Cancers derived from the target organs of GvHD such as colorectal cancer (*31*) and cholangiocellular carcinoma are also promising. Because allogeneic immune responses lead to epithelial damage of the gastrointestinal tract, colorectal cancer should reasonably respond to alloimmunity. Another supporting observation is that T-cell sensitization for GvHD occurs close to the Peyer's patch to induce strong allogeneic immune responses in the colon (*32*). Although few reports have been published on RIST for colorectal cancer, the report by Hentschke et al. (*31*) and the present study showed the association between GvHD and GvT effects in this cancer.

Third, we should establish the evaluation methods of treatment response after allo-SCT, which might be different from those after chemotherapy (*33*). The evaluation of immunotherapy frequently involves tumor markers. Because reliability of serum tumor markers is questionable (*34*), we recommend introducing the RECIST criteria to immunotherapy, thus enabling a comparison with chemotherapy. There are some controversies concerning the reliability of RECIST criteria in the response evaluation after cytotoxic chemotherapy against gastrointestinal tumors (*23, 35, 36*), and its application to immunotherapy requires careful consideration (*33*). In the RECIST criteria, pretreatment measurable lesions are identified, and their sum of longest diameters is compared before and after treatment. Even without enlargement of any of the measurable lesions, the emergence of a new lesion indicates PD, as shown in patient 4. This evaluation method is reasonable in chemotherapy, which has a prompt posttreatment response and transient effect. However, RIST requires several months until manifestation of efficacy, which may continue long after RIST (*15*). The longer duration until efficacy develops is problematic in progressive solid tumors. The tumor progression early after RIST occurs by nature, as shown in patient 3 and 4, until explicit treatment effect several months later. If the RECIST is applied in such cases, the evaluation results are PD. How can we evaluate cases with early progression and subsequent regression simultaneously with GvHD down to the preRIST size? Physicians involved in RIST have the impression that RIST has altered the natural disease progression and therefore has been effective. In contrast, most oncologists would believe PD. The integration of the evaluation concepts between RIST physicians and oncologists is an important and challenging issue. The ultimate goal of survival should be evaluated as a primary endpoint in a phase III trial.

Last, the development of tumor-specific treatments is warranted. At present, with the high probability of GvT effects in concert with GvHD, we permit GvHD symptoms to

TABLE 1. Tumor responses and outcomes

No	Age/Sex	Metastatic lesions	CEA (ng/ml)			CA19-9 (U/ml)			Tumor response according to RECIST[a]	Postmortem examination	Outcomes
			Pretransplant	Maximum	Minimum	Pretransplant	Maximum	Minimum			
1	44/M	Peritoneum	45.7	131	25.9	1388	2507	90	NA[b]	Reduction of peritoneal metastasis	Died of GVHD on day 62
2	52/F	Lung, liver	10360	10640	4175	5340	15150	3950	SD	NA	Died of PD on day 178
3	59/F	Lung, liver	48.6	181	12.4	38	70	23	PR	NA	Alive on day 390
4	52/F	Lung, liver, peritoneum, colon	9.3	14.2	6.8	158	529	219	PD[c]	Disappearance of lymph node metastasis. The other lesions remained stable[d]	Died of accident on day 88

[a] Tumor responses were defined according to the RECIST criteria. The lesions were evaluated monthly using CT scans.
[b] The patient had no measurable lesions.
[c] New lesions appeared in the chest CT on day 60, leading to the diagnosis of PD.
[d] The lesion of left supraclavicular lymph node shrunk with the development of acute GVHD. No lesion was found at postmortem examination.
GVHD, graft versus host disease; NA, not applicable; PR, partial response; PD, progressive disease.

some extent to maintain clinical efficacy of RIST. We may even modify the criteria for initiation of GvHD treatment (*37*). However, GvHD increases morbidity and mortality, leaving this method difficult in elderly patients (*38*). The problem might be overcome with an adjuvant tumor-specific immunotherapy (*39*), ex vivo priming of donor lymphocytes against tumor cells (*40*), use of highly immunosuppressive conditioning regimens (*41*), and the protection of GvHD target organs using cytokines (*42*).

In conclusion, the present study suggests the promising results of RIST in colorectal cancer. Because GvT effects are likely to be associated with GvHD, the optimization of conditioning regimens and GvHD management are necessary through phase II trials in colon cancer. Along with studies on GvT effects, the development of specific treatments, separate from GvHD, are needed.

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Research

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Clinical response in Japanese metastatic melanoma patients treated with peptide cocktail-pulsed dendritic cells

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Abstract

Background: Metastatic, chemotherapy-resistant melanoma is an intractable cancer with a very poor prognosis. As to immunotherapy targeting metastatic melanoma, HLA-A2$^+$ patients were mainly enrolled in the study in Western countries. However, HLA-A24$^+$ melanoma patients-oriented immunotherapy has not been fully investigated. In the present study, we investigated the effect of dendritic cell (DC)-based immunotherapy on metastatic melanoma patients with HLA-A2 or A24 genotype.

Methods: Nine cases of metastatic melanoma were enrolled into a phase I study of monocyte-derived dendritic cell (DC)-based immunotherapy. HLA-genotype analysis revealed 4 cases of HLA-A*0201, 1 of A*0206 and 4 of A*2402. Enriched monocytes were obtained using OptiPrep™ from leukapheresis products, and then incubated with GM-CSF and IL-4 in a closed serum-free system. After pulsing with a cocktail of 5 melanoma-associated synthetic peptides (gp100, tyrosinase, MAGE-2, MAGE-3 and MART-1 or MAGE-1) restricted to HLA-A2 or A24 and KLH, cells were cryopreserved until used. Finally, thawed DCs were washed and injected subcutaneously (s.c.) into the inguinal region in a dose-escalation manner.

Results: The mean percentage of DCs rated as lin$^-$HLA-DR$^+$ in melanoma patients was 46.4 ± 15.6 %. Most of DCs expressed high level of co-stimulatory molecules and type1 phenotype (CD11c$^+$HLA-DR$^+$), while a moderate number of mature DCs with CD83 and CCR7 positive were contained in DC products. DC injections were well tolerated except for transient liver dysfunction (elevation of transaminases, Grade I-II). All 6 evaluable cases except for early PD showed positive immunological responses to more than 2 melanoma peptides in an ELISPOT assay. Two representative responders demonstrated strong HLA-class I protein expression in the tumor and

very high scores of ELISPOT that might correlate to the regression of metastatic tumors. Clinical response through DC injections was as follows : 1 CR, 1 PR, 1 SD and 6 PD. All 59 DC injections in the phase I study were tolerable in terms of safety, however, the maximal tolerable dose of DCs was not determined.

Conclusions: These results suggested that peptide cocktail-treated DC-based immunotherapy had the potential for utilizing as one of therapeutic tools against metastatic melanoma in Japan.

Background

Despite many attempts in the last few years to target cancer-specific antigens, a breakthrough in terms of clinical response has yet to be achieved mainly because of a scarcity of effective genuine cancer antigens, immunological evasion, or an immunosuppressive state.

Melanoma-associated antigens are categorized as class I human leukocyte antigen (HLA)-restricted cancer/testis antigens [1] which are considered to be tolerable to the immune system because they are also expressed in normal tissues. However, malignant melanoma is the most well known cancer in which multiple tumor-specific antigens have been defined and utilized in vaccination strategies as peptide vaccines or peptide-pulsed DC vaccines [2-9].

From a clinical point of view, several vaccination strategies for stage IV melanoma using a combination of several (more than 3) peptides with a restriction to HLA-A2 have been reported to date [10,11]. However, little immunotherapeutic study regarding HLA-A24-restricted multiple peptides has been conducted because HLA-A24 is not a common allele in Caucasians. Several studies have demonstrated the identification of many HLA-A24-restricted CTL epitopes from various cancer-related antigens including p53, CEA, telomerase, tyrosinase, MAGE proteins etc. [12-18]. When it comes to melanoma, our group demonstrated the feasibility of using a combination of 5 melanoma-associated peptides with restriction of HLA-A24 (peptide cocktail) as a specific cancer vaccine in an immunotherapeutical trial (Akiyama et al, Anticancer Res., 2004). Based on basic research results, a phase I clinical trial of HLA-A2 or A24-restricted melanoma peptide cocktail-pulsed dendritic cell-based immunotherapy has been performed. Here we describe the safety and efficacy of DC-based immunotherapy against metastatic melanoma.

Materials and methods

Patient characteristics and eligibility criteria

Nine patients with metastatic melanoma were enrolled in a phase I clinical trial of a peptide cocktail-pulsed DC-based vaccine approved by the Institutional Review Board (No. 12–93 and 12–94) of the National Cancer Center, Tokyo. All patients gave written informed consent. All patients had received prior surgery, chemotherapy and radiation (Table 1). Three subjects had metastatic lesions in the brain and been given radiation to control them. Inclusion criteria were: i) biopsy-proven stage IV metastatic melanoma, ii) age ≥ 18 years, iii) performance status ≤ 2, iv) HLA-A2 or A24 phenotype and v) measurable target lesions. Exclusion criteria were : i) prior therapy < 4 weeks before trial entry, ii) untreated CNS lesion, iii) pregnancy, iv) autoimmune disease, and v) concurrent corticosteroid/immunosuppressive therapy. All the patients, who gave written informed consent, received subcutaneously (s.c) 3 DC vaccines at the inguinal region weekly and toxicity was checked. DCs were injected in dose-escalation design at a dose level per cohort of 1.0, 2.0 and 5.0 × 10^7/body/shot (Table 1). The injected DC number was calculated from the percentage of $Lin^-HLA\text{-}DR^+$ gated populations in a FACS analysis.

Table 1: Phase I study of DC-based therapy against melanoma

Patient No.	Age	Sex	Previous therapy	Measurable lesions	DC injection (times)	Side effect	DTH		Response
							peptide	KLH	
1	41	F	ST, CT, RT, IFNβ	lung, LN	1 × 10^7(10)	Hepatic (II)	-	++	PR
2	75	M	ST, CT, IFNβ	LN	1 × 10^7(10)	-	+	+	SD
3	49	F	ST, CT, IFNβ, RT	lung, liver	1 × 10^7(3)	-	-	-	(PD)*
4	49	M	ST, CT	lung, liver	2 × 10^7(6)	-	-	-	PD
5	50	M	ST, CT, IFNβ	lung, liver, LN	2 × 10^7(6)	Hepatic (I)	-	-	PD
6	69	M	ST, CT, IFNβ	LN	2 × 10^7(10)	-	+	+	CR
7	61	M	ST, CT, RT	liver, LN	5 × 10^7(8)	Hepatic (I)	+	++	PD
8	64	F	ST, CT, RT	lung	5 × 10^7(3)	Fever (I)	-	-	(PD)
9	66	F	ST, CT,	lung, LN	5 × 10^7(3)	-	-	-	(PD)

* The (PD) patients represent those who received fewer than 4 DC injections because of an early progression of the disease.

Preparation of DCs and peptides

Leukapheresis products from 7 L of processed blood were washed and centrifuged using density-adjusted OptiPrep™ (Axis-Shield PoC, Oslo, Norway), then the monocyte layer at the top was retrieved. Cells were transferred to an X-fold culture bag (Nexell, Irvine, CA) and cultured in the presence of GM-CSF at 50 ng/ml (CellGenix, Freiburg, Germany) and IL-4 at 50 ng/ml (CellGenix) in X-VIVO15 serum-free medium (Biowhittaker, Walkersville, MD). After 7 days, harvested cells were pulsed with a cocktail of 5 melanoma-specific synthetic peptides (25 μg/ml each) restricted to HLA A2 or A24 and KLH (25 μg/ml, Intracell, Frederick, MD). DC-enriched cells were washed and cryopreserved in Cryocyte bags (Baxter Healthcare Co., Deerfield, IL) until used. The purity of $CD14^+$ cells was evaluated with a flow cytometer (FACSCalibur, Becton-Dickinson Co., CA) before and after OptiPrep™ separation. The percentage of DCs was rated as the $lin^-HLA\text{-}DR^+$ population (lineage antibodies including CD3, CD14, CD16, CD19, CD20, CD56 ; Becton-Dickinson Co.). The additional DC-related markers were determined on gated $lin^-HLA\text{-}DR^+$ cells. The following peptides restricted to HLA-A2 or A24 were synthesized according to GMP standards by Multiple Peptide Systems, CA. HLA-A2: MART-1_{27-35} (AAGIGILTV), $gp100_{209-217}$ (IMDQVPFSV), $tyrosinase_{368-376}$ (YMDGTMSQV), MAGE-$2_{157-166}$ (YLQLVFGIEV), MAGE-$3_{271-279}$ (FLWGPRALV) ; HLA-A24: $gp100_{152-160}$ (VWKTWGQYW), $tyrosinase_{206-214}$ (AFLPWHRLF), MAGE-$1_{135-143}$ (NYKHCFPEI), MAGE-$2_{156-164}$ (EYLQLVFGI), MAGE-$3_{195-203}$ (IMPKAGLLI).

Characterization of tumor specimens before DC vaccines

Skin metastatic lesions were obtained from patients who gave written informed consent. The expression of melanoma tumor antigens was investigated using RT-PCR as described previously [19]. HLA protein expression was also evaluated using an immunohistochemical (IHC) analysis with anti-HLA-A2 or A24 monoclonal antibody (One Lambda Inc., Canoga Park, CA). A phenotypical analysis of lymphocytes infiltrating the tumor site was also performed using IHC.

Clinical and immunological monitoring

Adverse effects were evaluated according to the NCI Common toxicity criteria after 3 DC injections. Standard conventional definitions of major (complete or partial) objective responses were used. Stable disease (SD) was defined as less than a 25% change in size with no new lesions lasting at least 4 weeks. Clinical response was rated as maximal through the DC vaccinations. The patients received up to 10 injections on the condition that at least one measurable lesion showed more than stable disease (SD) response and/or an ELISPOT assay performed after 4 injections indicated a positive response for more than 1 melanoma-associated peptides. PBMC samples were harvested before and 29, 78, 134 and 190 days after the 1 st DC injection, and frozen prior to use for immunological monitoring tests. All patients were followed up for 2 years after the enrollment into the study.

ELISPOT assay

The ELISPOT assay was performed using in vitro re-stimulations. Briefly, PBMCs were incubated in a 24-well culture plate at 4×10^6 per ml and divided into non-adherent and adherent cells. Adherent cells were treated with a peptide cocktail and β2-microglobulin for 2 hrs, and co-cultured with non-adherent cells in the presence of IL-2 at 15 U/ml and IL-7 at 10 ng/ml. On day 7, non-adherent cells were re-stimulated with peptide-pulsed adherent cells. On day14, responder cells (1×10^4/well) were incubated with peptide-pulsed target cells (1×10^5/well; .221A201 cells for HLA-A2 peptide or TISI cells for HLA-A24 peptide) in a 96-well culture plate coated with anti-IFN-γ antibody (MABTECH AB, Nacka, Sweden) overnight. Finally positive spots stained with anti-IFN-γ antibody were measured using the KS ELISPOT system (Carl Zeiss AG, Overkochen, Germany). HLA-A2-restricted Influenza M1 peptide (GILGFVFTL) or HLA-A24-restricted EBNA3A peptide (RYSIFFDY) was used as a negative control.

Tetramer staining

PBMCs were re-stimulated twice in vitro and utilized for tetramers staining. $CD8^+$-enriched T cells were obtained by the depletion of $CD4^+$ T cells using Dynabeads M-450 CD4 (Dynal, Oslo, Norway) and used for tetramers staining. The staining was performed according to the method reported by Kuzushima et al [20]. The PE-labeled tetramers used in the present study were as follows: HLA-A*0201 MART1 (Beckman Coulter Inc., San Diego, CA), HLA-A*0201 gp100, HLA-A*2402 tyrosinase, HLA-A*2402 MAGE-1, HLA-A*2402 HIV (RYLRDQQLL) and HLA-A*0201 Influenza M1 tetramers (MBL, Nagoya, Japan).

Intracellular cytokine staining

PBMCs were stimulated with 25 ng/ml of PMA (Sigma) and 1 μg/ml of ionomycin (Sigma) for 5 hrs in a 96-well culture plate. Breferdin A (10 μg/ml) was also added to cultures in the last hour. After the stimulation, cells were stained with FITC-anti-CD4 MoAb, and subsequently intracellular staining was performed with fix/permealization buffer and PE-labeled anti-IFN-γ or anti-IL-4 MoAb (Pharmingen, San Diego, CA). Finally, the ratio of Th1 ($IFN\text{-}\gamma^+$) and Th2 ($IL\text{-}4^+$) was calculated in PBMC samples obtained before and after DC vaccination.

DTH reactions

The HLA-A2 or A24 peptide cocktail solution diluted to a dose of 5 μg/ml (each peptide) and KLH (50 μg/ml) were injected intradermally on the patient's forearm and the

Table 2: Immunological monitoring in melanoma patients

Patient No.	HLA	Tumor antigen, HLA expression	ELISPOT	Tetramer	Th1/Th2 balance
1	A*2402	3/5(Tyr,M1,M2), A24(+)	3/5(Tyr,M1,M2)	Tyrosinase (0.34%)	5.19 (1.45)[a]
2	A*0201	A2(+)	2/5(MART1,gp100)	MART1 (0.64%)	3.68 (1.49)
3	A*2402	A24(-)	N. D[b]	N. D.	-
4	A*0206	A2(-)	2/5(MART1, M2)	-	3.05 (2.57)
5	A*0201	A2(-)	2/5(MART1, M2)	MART1 (1.48%)	2.83 (3.68)
6	A*2402	2/5(M2,M3), A24(+)	2/5(M2, M3)	-	3.76 (2.00)
7	A*0201	4/5(MART1,Tyr,gp100,M2), A2(+)	2/5(gp100, M2)	-	2.64 (1.79)
8	A*2402	A24(-)	N. D.	N. D.	-
9	A*0201	A2(+)	1/5(gp100)[c]	N. D.	N. D.

[a]The value in the parenthesis shows Th1/Th2 ratio prior to DC vaccination. [b]N. D. ; not done.
[c]The value shows the one obtained prior to DC vaccines.

redness and induration at the injection site was measured. PPD was used as a positive control.

Statistical analysis

Statistical differences were analyzed using Student's paired two-tailed *t*-test. Values of $p < 0.05$ were considered significant.

Results

DC characterization

The mean percentage of DCs rated as lin$^-$HLA-DR$^+$ in melanoma patients was 46.4 ± 15.6 %, not different from that in healthy volunteers (data not shown). The frequencies of the DC-related markers were determined on gated lin$^-$HLA-DR$^+$cells : HLA-class I 97.5 ± 0.9 %, CD80 87.6 ± 6.9 %, CD86 85.5 ± 7.4 %, CD1a 55.2 ± 24.2 %, CD83 29.9 ± 13.3 %, CCR7 32.4 ± 13.7 %, DC SIGN 78.2 ± 19.3 %, CD11c$^+$HLA-DR$^+$ 90.6 ± 6.0 %, CD123$^+$HLR-DR$^+$ 0.99 ± 1.3 %. Most of DCs expressed high level of co-stimulatory molecules and type1 phenotype (CD11c$^+$HLA-DR$^+$), while a moderate number of mature DCs with CD83 and CCR7 positive were contained in DC products. On the other hand, the T cell-stimulating activity of DCs investigated in the MLR assay using allogeneic T cells was as strong as that of DCs obtained from healthy volunteers (data not shown).

Characterization of tumor specimen

An analysis of melanoma antigen expression by RT-PCR was performed in 3 cases. The expression of more than 2 antigens in the tumor was verified in all cases. HLA protein expression was positive in 5 out of 9 cases (Table 2). Patient 1 who showed a remarkable clinical response (PR), was representative of HLA protein-positive cases (Fig. 1). In contrast, in patient 7, HLA-A2 protein expression in the tumor was lost in the course of treatment.

ELISPOT assay

CTL precursors of more than 2 melanoma peptides were recognized after DC vaccines in 6 of 9 cases. Two HLA-A2$^+$ cases (patients 5 and 9) showed HLA-A2 peptide-specific CTL responses before the vaccination. Patients 1 and 6, which showed remarkable clinical responses, exhibited many CTL precursors against a HLA-24 restricted peptide-cocktail (Table 3, Figure 2). Notably, in patient 1, a remarkable increase in the CTL response to the HLA-A24 peptide cocktail was seen in accordance with the regression of metastatic tumor of the lung (Fig. 3). On the other hand, patient 7 also demonstrated a high CTL precursor frequency, but showed no significant clinical response.

Tetramer staining

After CD4$^+$ T cell depletion, the frequency of CD8$^+$ cells was more than 85%. The proportion of PE-labeled tyrosinase-HLA-A24 tetramer-positive cells among gated CD8$^+$ cells was 0.34% in patient 1 (Table 2). HIV-A24 tetramer (negative control)-positive cells were not detected. The percentage of PE-labeled MART1-HLA-A2 tetramer-positive cells was 0.64% and 1.48% in patients 2 and 5, respectively. On the other hand, that of Influenza M1-HLA-A2 tetramer (negative control)-positive cells was 0.04%.

Th1 and Th2 balance after DC vaccination

In 5 of 6 evaluable cases, the balance of Th1 and Th2 shifted more to Th1 after 4 DC injections compared with prior to vaccination. (Table 2). The amplitude of the shift seemed to be larger in clinical responders (patients 1, 2, 6) than non-responders (patients 4, 5, 7) (% of ratio increase; 264 ± 86 vs. 114 ± 35).

DTH

Three of 6 evaluable cases showed positive DTH to a peptide-cocktail after DC injections (Table 1). On the other hand, 4 of 6 cases developed a DTH response to KLH pro-

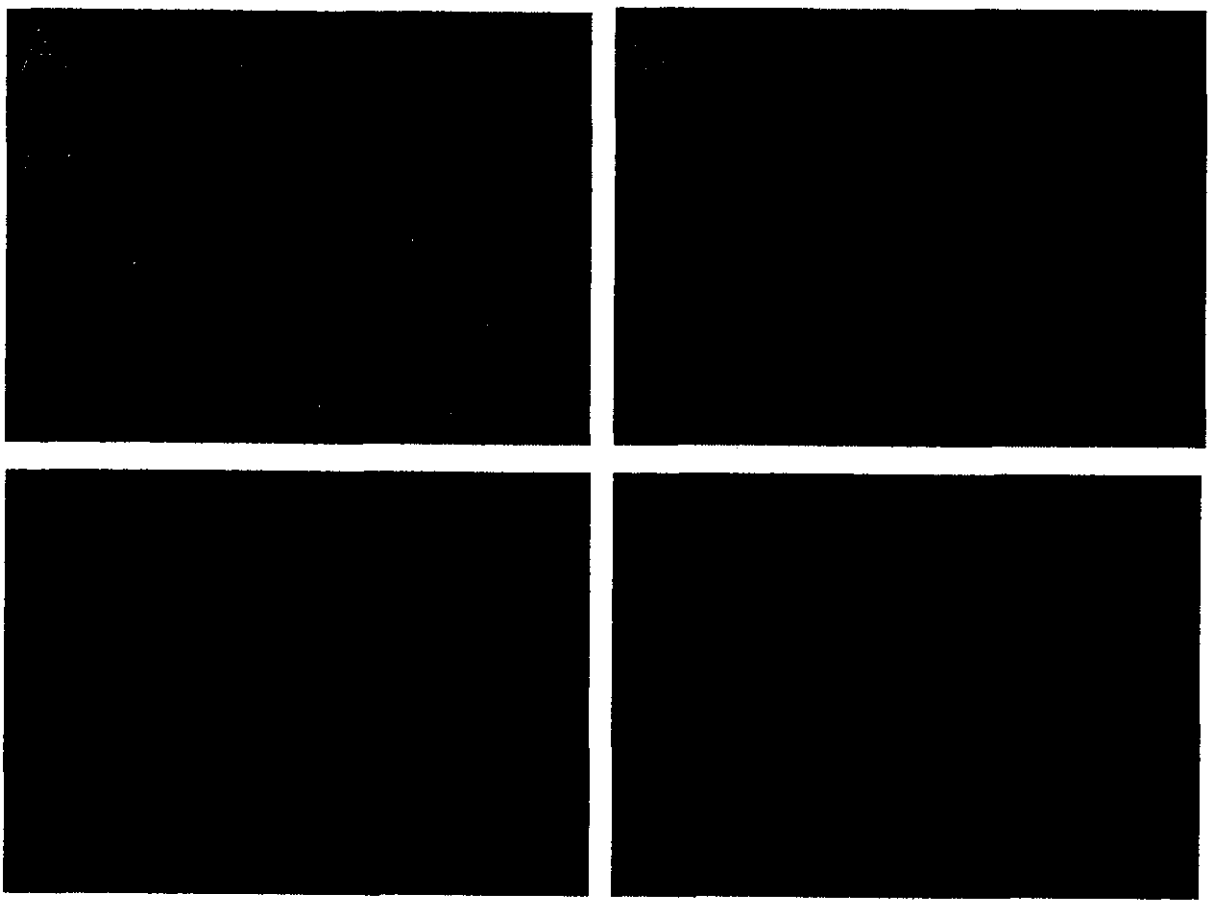

Figure 1
Immunohistochemical analysis of metastatic tumor tissue from responder patient 1 and non-responder patient 7. A; H-E stain and B; anti-HLA-A24 MoAb from patient 1. C; anti-HLA-A2 MoAb before DC vaccination and D; anti-HLA-A2 MoAb after 4 DC injections from patient 7. Magnification × 200.

tein. There were stronger reactions to KLH in patients 1 and 7.

Adverse effects of DC vaccine

Safety was assessed after 3 DC injections in all 9 cases. Three of 9 patients developed mild hepatic dysfunction (grade I-II), however it was only transient and disappeared in spite of the continuance of DC injections. Rheumatoid factor and anti-nuclear antibody were negative before the injection, but increased to 1:160 and 1:40, respectively after the injections finished in patient 1. No clinical symptoms of autoimmune disease were found in patient 1 (Table 1).

Clinical response

Clinical response was rated as maximal through the DC vaccinations. In 6 evaluable cases except for 3 cases of early PD cases due to a rapid progression of the disease, 1CR (patient 6), 1PR (patient 1), 1SD and 3 PD were obtained (Table 1). Large metastatic lesions in the lung and hilar nodes in patient 1 dramatically decreased in size after 4 DC injections, and almost disappeared after treatment finished (Fig. 3). Moderate sized cervical metastatic lesions in patient 6 finally started to decrease after 8 DC injections and disappeared surprisingly rapidly after the finish of DC therapy. In contrast, patient 7 who exhibited good immunological responses in the ELISPOT assay and

Table 3: Peptide cocktail-specific CTL precursor frequency during DC vaccination

Patient No.	DC injection (times)	Spot No./CD8+ T cell (%)[a] before	day29	day78	day134	day190
1	1×10^7(10)	1.19/0.45	6.96/0.06	8.82/0.63	8.81/0.08	5.4/0.08
2	1×10^7(10)	0.07/0.05	0.07/0.2	0.02/0	0.02/0	0.29/0.03
3	1×10^7(3)	N.D.[b]	N.A.[c]	N.A.	N.A.	N.A.
4	2×10^7(6)	0.39/0.53	1.29/0.03	1.12/0	N.A.	N.A.
5	2×10^7(6)	1.74/0.05	0.51/0.2	1.25/0.04	N.A	N.A.
6	2×10^7(10)	0.21/0.27	0.31/0.28	1.18/0.24	7.80/0.19	9.82/0.30
7	5×10^7(8)	0.62/0.20	6.52/0.1	7.33/0.11	N.A.	N.A.
8	5×10^7(3)	N.D.	N.A.	N.A.	N.A.	N.A.
9	5×10^7(3)	3.09/1.24	N.D.	N.A.	N.A.	N.A.

The percentages represent IFN-γ-positive spot No. divided by total CD8+ cell No. from 1×10^4 PBMCs. [a]Each value represents the percentage with peptide cocktail/without peptide cocktail. [b]N.D. ; not done, [c]N.A. ; sample not available.

DTH, showed no shrinkage of the tumor, resulting in cessation after 6 DC injections.

Characterization of infiltrated lymphocytes in the tumor

IHC analysis of infiltrated lymphocytes in the tumor after DC vaccines was performed only in patient 1 and 7. The obvious infiltration of a larger number of CD4+ or CD8+ T cells and a small number of CD20+ B cells were shown in patient 1 (Fig. 4). In contrast, no significant cell infiltration was seen in patient 7 who did not develop any therapeutical effect on the tumor (data not shown).

Discussion

Clinical trials of specific immunotherapy against metastatic melanoma using peptide-pulsed Mo-derived DCs have been performed in mainly Western countries, and some fruitful results were obtained [7,10,11]. In those cases, most of the patients belonged to the HLA-A*0201 type. In the present study, we investigated the effect of peptide-pulsed DCs on 4 cases of HLA-A*2402+ metastatic melanoma patients besides 4 cases of HLA-A*0201+ patients in a clinical phase I trial. This is the first report to demonstrate that peptide-pulsed DCs were effective in some HLA-A24+ melanoma patents in Japan. It is well known that HLA-A*2402 is a common genotype and around 60% positive in Asians. There was one case of HLA-A*0206 patient among 5 HLA-A2+ patients (Table 2). Sidney et al. [21] demonstrated that over 70% of the peptides that bound A0201 with high affinity were found to bind at least two other supertype molecules like A*0202, A*0203 or A*0206. Taking it into considerations, the HLA-A*0206 patient was finally enrolled into the study. With regard to other HLA-A24+ solid cancers, stomach, colon and bladder cancers have been treated with peptide (MAGE-3)-pulsed DC vaccines, and showed a limited response [22-24]. Considering that melanoma is highly immunogenic and probably a good model for tumor-specific immunotherapy despite being an unusual tumor in Asian countries, it deserves a phase I study using peptide-pulsed DCs.

In our study, peptide cocktails combining 5 peptides for each HLA type (HLA-A2 or A24) were prepared and used for DC pulsing. Our clinical study revealed positive ELISPOT responses against more than 2 peptides in all 6 evaluable cases. In previous reports, clinical DC therapy using more than 3 melanoma peptides demonstrated the induction of a specific CTL response against multiple melanoma peptides [10,11]. However,, there is still some controversy over the efficacy of multiple epitope-based vaccinations and Smith *et al.* [25] demonstrated that, although polyepitope vaccines are an effective way of priming polyvalent CTLs, continual stimulation with polyepitope vaccines might restrict CTL induction as a result of immunodominance. The results of our study are thought to answer that question, but testing of the peptide cocktail vaccine in more patients will be needed.

To refine the quality and protocol of the tumor-specific immunotherapy for clinical trials, the prediction of clinical response in an individual is important [26] and should be discussed. In our study, the correlation between immunological parameters and clinical response was investigated in a limited number of cases.

First of all, as to HLA expression in the tumor, patients 1, 2, 6 and 7 were positive, and patients 4 and 5 were negative. HLA-negative cases showed a progression of the tumor. Even in positive cases, patient 7 turned negative in the course of DC therapy, showing tumor progression.

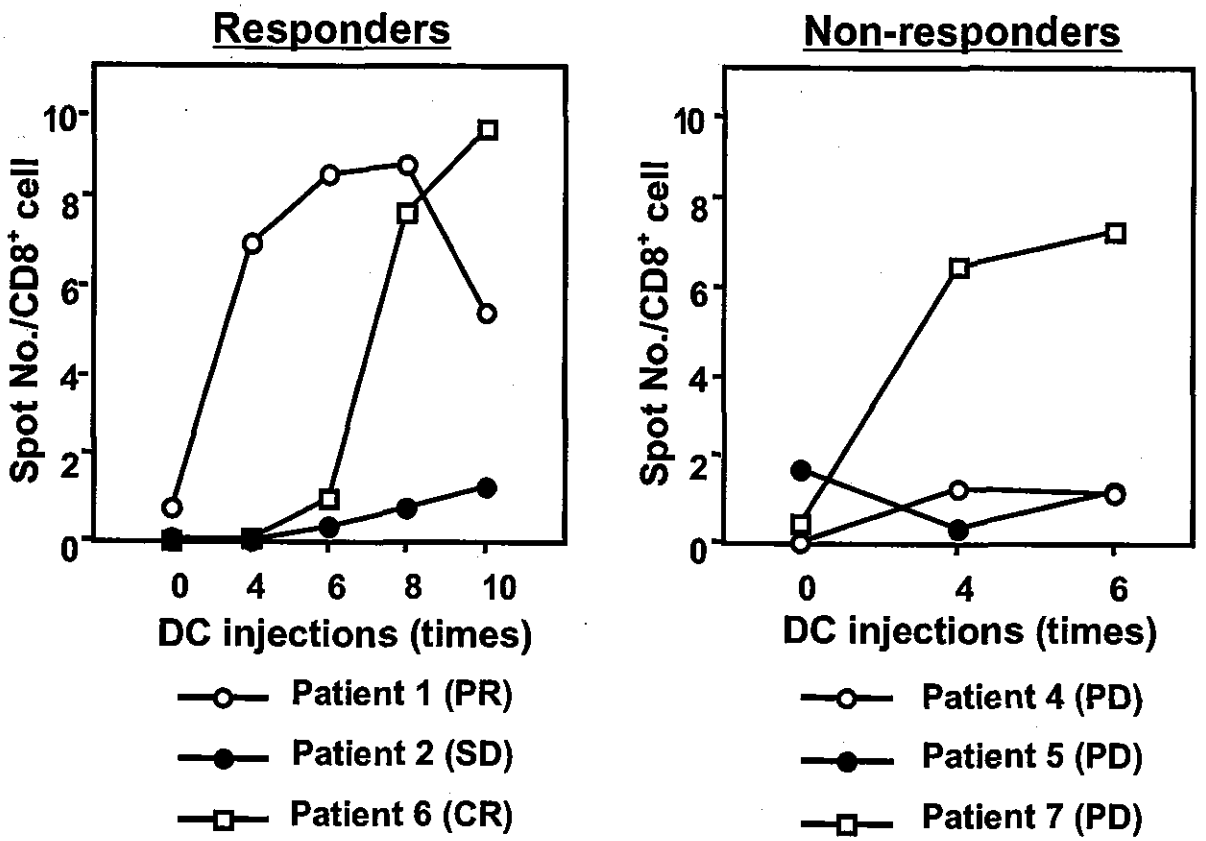

Figure 2
CTL responses in the course of DC injections in 6 evaluable cases. Patients 1, 2 and 6 were responders and patients 4, 5 and 7 were non-responders. Responders (cases 1,6) showed remarkable CTL expansion in PBLs compared with before DC vaccination. In contrast, non-responders (patients 4,5) showed no significant CTL responses except in patient 7.

Loss of HLA expression in melanoma is reported to be a complex phenomenon associated with melanoma antigen loss [27], β2-microglobulin gene mutation [28] or loss of heterozygosity (LOH) in chromosome 6 and may lead to tumor progression and metastasis. As to patient 7, considering that the melanoma antigen expression was maintained, the functional expression of β2-microglobulin should be investigated. All the other HLA-positive cases showed CR, PR and SD, respectively. There was a tendency for HLA expression to be associated with tumor response, and some researchers reported a positive correlation of HLA-expression to tumor response in immunotherapy against melanoma. However, despite the positive correlation of HLA-expression in the tumor with antitumor response, Nestle et al. demonstrated that HLA-expression in the tumor did not correlate to survival in melanoma patients [29].

Second, the amplitude of the CTL response in the ELISPOT assay seems to be another key factor predicting antitumor response. Patients 1, 6 and 7 showed large responses to peptide cocktail in ELISPOT, and patients 2, 4 and 5 showed small responses. The former exhibited a remarkable regression of tumor except patient 7. On the other hand, the latter showed a poor response. There was a likely tendency that the amplitude of the CTL response was associated with tumor regression. Also, it was difficult to predict when immunological responses like CTL induc-

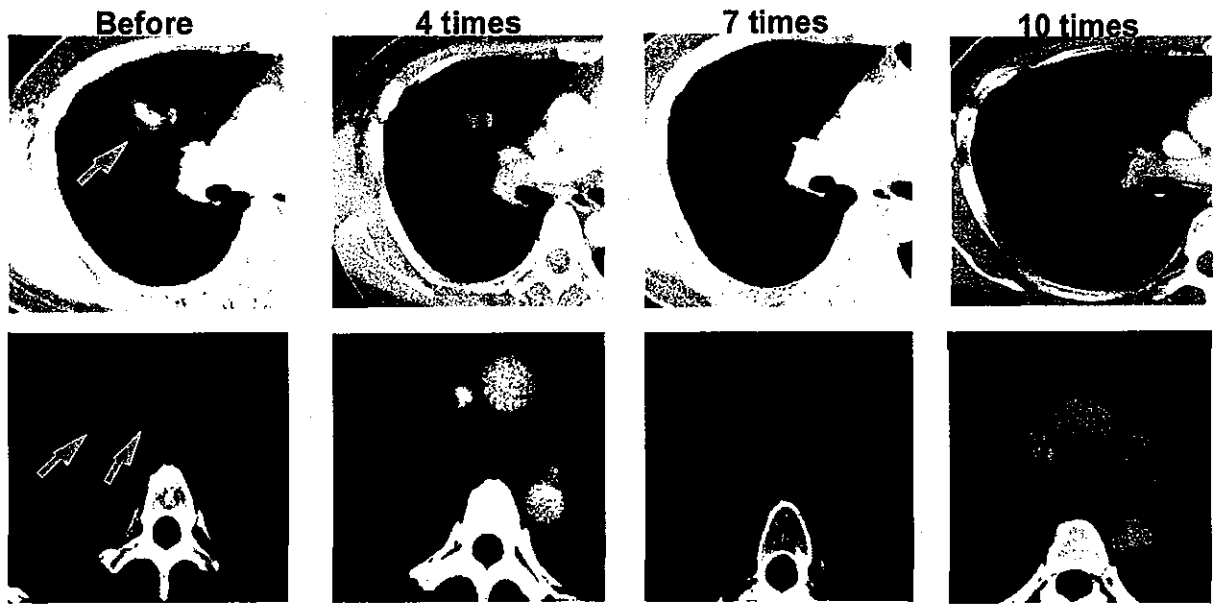

Figure 3
Impact of DC vaccines on metastatic lesions of the lung in responder patient 1. Upper and lower panels show a lung and hilar lymph node metastatic lesion (arrow), respectively. The CT scan was made before therapy and after 4, 7 and 10 DC vaccinations.

tion start to be activated in vivo during DC vaccination, and this question needs to be answered. In the present study, because of a limited number of patients given DC vaccines, the tendency that HLA-class I protein expression in the tumor and the amplitude of ELISPOT responses are seemingly associated with tumor regression is not convincing.

Finally, in order to improve tumor response in the present study, there are still some issues regarding clinical DC preparation. First of all, the purity of $CD14^+$ cells after Opti-prep separation is still low and may not be reproducible. Therefore, other clinical grade-monocyte separation methods using an elutriator or negative selection with CD2 and CD19 MoAbs [30] should be tried. Second, considering that the amplitude of the CTL response was associated with tumor regression, and that even a remarkable increase of CTL frequency inevitably diminished in spite of the repetition of DC vaccinations, it seems to be crucial to maintain increased CTL frequency in blood leading to TIL in the tumor and expand more than enough to develop a substantial number of memory $CD8^+$ CTL in lymph nodes. Such a novel method will be needed to develop an effective cancer vaccine.

Conclusions

In the present study, we investigated the effect of dendritic cell (DC)-based immunotherapy on metastatic melanoma patients with HLA-A2 or A24 genotype. Nine cases of metastatic melanoma were enrolled into a phase I study using HLA-A2 or A24-restricted peptide cocktail-pulsed DCs. All 6 evaluable cases showed positive immunological responses to more than 2 melanoma peptides in an ELISPOT assay. Clinical response through DC injections was as follows : 1CR, 1 PR, 1SD and 6 PD. All 59 DC injections in the phase I study were safely administered to patients. These results suggested that peptide cocktail-treated DC-based immunotherapy had the potential for utilizing as one of therapeutic tools against HLA-A2 or $A24^+$ metastatic melanoma.

Abbreviations

DC, dendritic cell ; HLA, human leukocyte antigen ; GM-CSF. granulocyte macrophage-colony-stimulating factor ; IL, interleukin ; KLH, Keyhole limpet hemocyanin ; CTL, cytotoxic T cell ; DTH, delayed-type hypersensitivity ; CR, complete remission : PR, partial remission ; SD, stable disease ; PD, progressive disease ; RT-PCR, reverse transcription-polymerase chain reaction ; IFN, interferon ; PBMC, peripheral blood mononuclear cell.

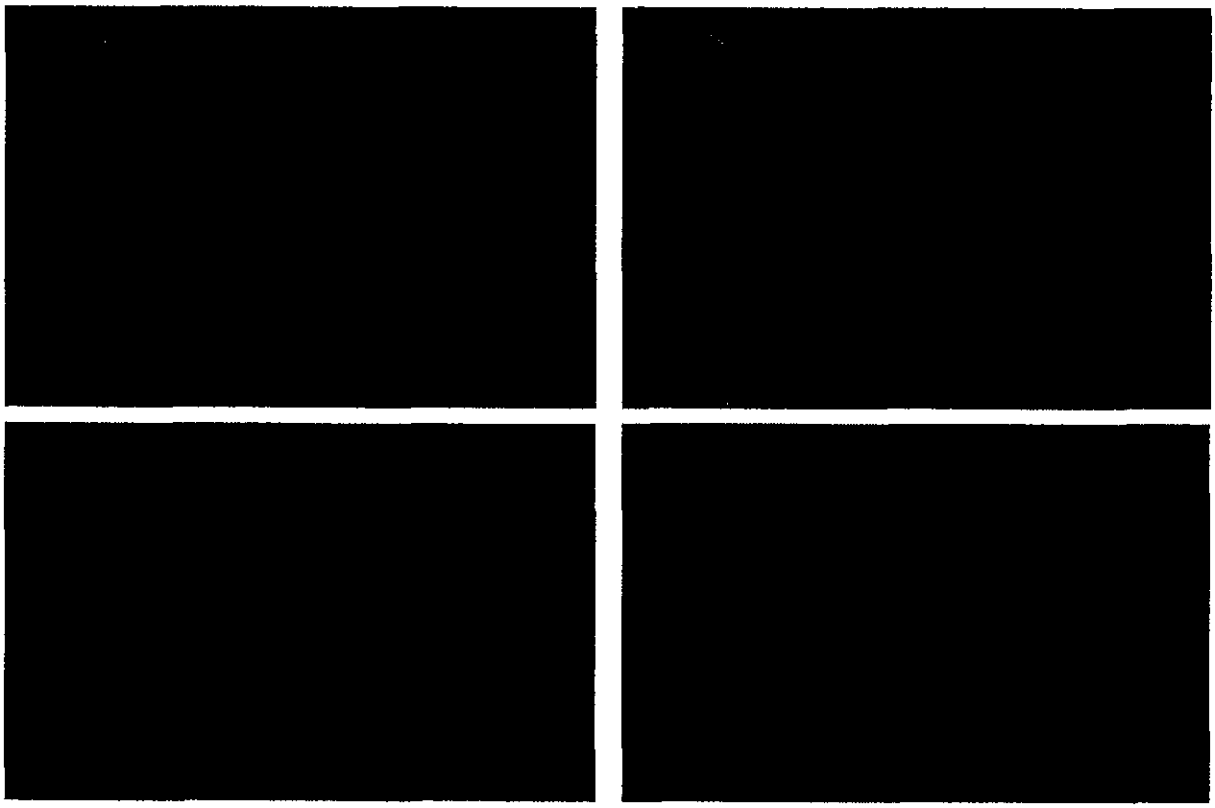

Figure 4
Phenotype analysis of lymphocytes infiltrating the tumor site in responder patient 1. Obvious infiltration of a larger number of $CD4^+$ or $CD8^+$ T cells and a small number of $CD20^+$ B cells is shown. Indirect staining using anti-CD4, CD8, CD20 or CD56 MoAb as primary Ab and goat anti-mouse Ab as secondary Ab was performed. Magnification × 200.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
YA participated in the design of the study and drafting the manuscript and were responsible for completing the study. RT, NI, MS, YH carried out apheresis and cell processing and were responsible for DC production. AY and NY were responsible for the clinical side of the study. IK, IN, KT and KM participated in the design of the study and performed biological assays. YT and KY reviewed the manuscript. All authors read and approved the final manuscript.

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