Table I. Patient characteristics and clinical outcomes of CEA-DC vaccine therapy (October, 1998-June, 2003).

Primary disease	Patient no.	Age/Sex	Metastasis	No. of vaccination	Serum CEA (ng/ml)	After therapy	Survival[a]
Colorectal cancer	F47	65/F	Liver, lung	5	↑	-	4
	F43	64/F	Liver, LN	5	↑	-	3
	F42	61/F	Liver	6	↑	-	7
	F49	51/M	Liver	6	↑	Chemo	25
	F102	71/F	Peritoneum	6	↑	-	7
	F23	54/F	Lung, pelvis	7	↑	-	4
	F38	28/F	Peritoneum	8	↑	-	6
	F32	56/M	Liver	10	↑	-	8
	F45	46/F	Bone	12	↓↑	-	8
	F40	42/F	Liver	15	→↑	Chemo	18
	F27	57/F	Lung, adrenal	39	→	Chemo	31
Gastric cancer	F41	33/F	Peritoneum	8	→	-	6
	F46	53/M	Lung, brain	8	↑	-	6
Lung cancer	F48[b]	56/M	Bone, LN, (brain)	10	↓(23.6→2.5)	Radio, Chemo, Surg	Alive (46)
	F103	57/M	Bone, brain	12	↓↑	Chemo	14
	F101	39/M	Bone	17	↓(30.9→4.6)	Unknown	Unknown
	F44	69/M	Pleura	22	↑	-	13
	F37	52/F	Bone	22	↓(394→42)	-	14

[a]Survival is defined as months to death after the beginning of CEA-DC vaccine therapy. LN, lymph node; Chemo, chemotherapy; Radio, radiotherapy; Surg, surgery. [b]Case F48 developed a solitary brain metastasis after 10 times of CEA-DC vaccination with a decrease of serum CEA level. Afterwards, he received radiotherapy, chemotherapy and surgical resection of the brain metastasis, and he is still alive at the time of writing.

Dendritic cells (DCs) are professional antigen-presenting cells that can induce a strong T cell-mediated immune response. They can be induced *in vitro* from peripheral blood monocytes in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (4). DCs have high expression levels of adhesion molecules and costimulators on their surface and migrate to lymphoid organs, where they activate antigen-specific T cells. Because DCs stimulate both memory and naive T cells, DC-based immunotherapy is considered a promising approach for cancer treatment (5).

In this study, active specific immunotherapy using autologous DCs pulsed with CEA652 was used to treat HLA-A24-positive patients who had advanced gastrointestinal or lung adenocarcinomas that expressed CEA. Eighteen patients with metastatic lesions refractory to conventional therapies were enrolled. No treatment-related toxicity occurred, and some patients showed an immune response to CEA652 peptide as well as a clinical response to treatment.

Patients and methods

Patients. Eighteen patients were enrolled from December 1998 through December 2001 (Table I). All patients had metastatic lesions from gastrointestinal or lung adenocarcinomas that were refractory to conventional chemotherapy or radiotherapy. The study protocol was approved by the Institutional Ethics Review Committee, Kyoto Prefectural University of Medicine, and written informed consent was obtained from all subjects at the time of enrollment. The protocol required that all patients: a) were positive for HLA-A24(2402); b) had metastatic lesions expressing CEA as confirmed by immunohistochemical analysis or elevated serum CEA levels; c) had an ECOG (Eastern Cooperative Oncology Group) performance status of 0 or 1; and d) had adequate cardiac, pulmonary, hepatic, renal, and hematological function. Patients were excluded if they: a) had received chemotherapy, radiotherapy, or immunotherapy within 4 weeks before enrollment; b) had severe infectious, hematological, cardiac, or pulmonary disease; c) had autoimmune disease; d) were receiving steroid therapy; or e) were pregnant.

Generation of peptide-pulsed DCs. Patients underwent leukapheresis with an exchange of approximately 10 l of blood with the use of a Blood Cell Separator CS-3000 (Baxter, Seattle, USA) to obtain peripheral blood mononuclear cells (PBMCs) after mobilization by recombinant human granulocyte-colony stimulating factor (G-CSF) [Gran (filgrastim); Kirin Brewery, Gunma, Japan], administered at a dose of 5-10 μg/kg once daily for 5 consecutive days. The leukapheresis products were separated by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Stockholm, Sweden). The resulting

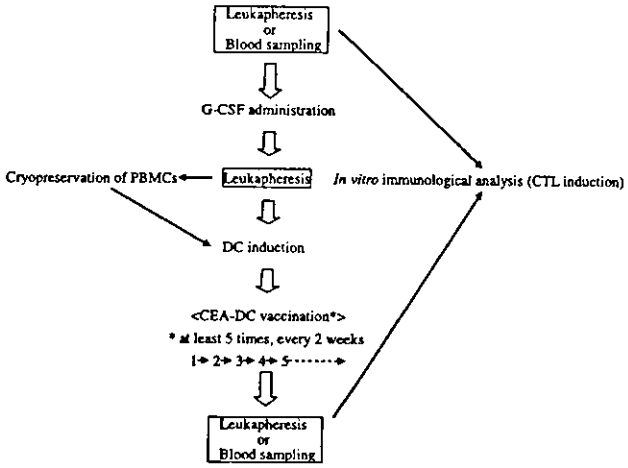

Figure 1. Treatment protocol. DCs for clinical use were induced *in vitro* from G-CSF-mobilized PBMCs in the presence of GM-CSF and IL-4. *In vitro* immunological analysis was done using PBMCs obtained by leukapheresis or blood sampling before and after CEA-DC vaccination.

PBMCs were suspended in CP-1 (Kyokuto Seiyaku, Tokyo, Japan) mixed with an equal volume of RPMI 1640 (Nikken, Kyoto, Japan) at about $5x10^7$ cells/ml, and the cell suspension was divided into vials containing 2 ml each. All vials were stored in a liquid nitrogen tank. Two or three vials were used to prepare DC vaccine for each treatment. After thawing, the PBMCs were suspended in 20 ml of complete medium [RPMI 1640 supplemented with 5% heat-inactivated pooled human AB plasma (FFP; Japanese Red Cross Society)] and gentamycin (Fujisawa Pharmaceutical, Osaka, Japan), and the cell suspension was plated in 75-cm^2 cell culture flasks. The flasks were incubated in a 5% CO_2 incubator at 37°C for 2 h, and non-adherent cells were removed. Adherent cells (monocyte-enriched fraction) were cultured in 30 ml of complete medium containing 1,000 U/ml each of recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF; Kirin) and recombinant human interleukin-4 (IL-4; Genzyme, Minneapolis, MN). After culturing for 7 days, DCs were harvested, washed twice, and suspended in 2 ml of phosphate-buffer saline (PBS) containing 1% human albumin (Fujisawa). CEA652 peptide, TYACFVSNL (Takara, Shiga, Japan), was dissolved at 40 µg/ml and added to the DC suspension at room temperature. After 4 h, the cells were washed and prepared as a cell suspension in 2 ml of saline containing 1% human albumin. The cell suspension was used as the vaccine (CEA-DC vaccine) (Fig. 1).

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

Patient treatment. Patients received vaccination on an outpatient basis and were monitored for acute toxicity at least 3 h after treatment in the hospital. The standard vaccination schedule was as follows. DC vaccine preparation (0.5 to $5x10^7$ cells; 1 ml/dose) was injected intradermally and subcutaneously at the same site in the inguinal region via a 26-G needle. Treatment consisted of at least 5 vaccinations at 2-week intervals. During the vaccination period, patients received no other treatment for their cancer, and blood samples were drawn to monitor serum CEA levels every 2 weeks. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria. Radiography, computed tomography (CT), and magnetic response imaging (MRI) were conducted at 4- to 8-week intervals to evaluate the development of metastatic lesions. For *in vitro* immunological analysis, if physical conditions permitted, patients underwent leukapheresis with an exchange of approximately 5 l of blood within 1 week before the onset of G-CSF mobilization and 2 weeks after the fifth vaccination to collect PBMCs, which were frozen and stored in liquid nitrogen until use (Fig. 1).

If the treatment was judged to be ineffective after at least 5 vaccinations, patients in relatively good physical condition were permitted to receive chemotherapy with new drugs [irinotecan hydrochloride (CPT-11), gemcitabine hydrochloride (GEM), etc.] or radiotherapy.

Delayed-type hypersensitivity skin test. To monitor delayed-type hypersensitivity (DTH) response, skin tests were performed before the first and after the fifth vaccination in each patient. A positive skin-test reaction was defined as skin erythema and induration >5 mm in diameter 48 h after injection. DCs ($1x10^5$ cells) pulsed with CEA652 at a concentration of 40 µg/ml were injected in alternate sides of the inguinal region. As control, DCs without peptide were tested simultaneously. Patients returned to the hospital 2 days after vaccination, and the diameter of the local skin erythema and induration was measured.

In vitro assessment of antigen-specific CTL induction. To determine the immune response to CEA652 peptide *in vitro*, peptide-specific CTL generation from PBMCs obtained before and after vaccination was semi-quantitatively assessed by ^{51}Cr release assay. CTL induction was carried out with the use of peptide-pulsed DCs as described previously (5). Briefly, $CD8^+$ T cells in PBMCs harvested before and after vaccination by leukapheresis were cultured *in vitro* with irradiated peptide-pulsed DCs. The cultures were performed in 48-well plates. On days 7 and 14, the T cell cultures were restimulated with peptide-pulsed adherent monocytes. After four cycles of restimulation, each well was tested separately on day 36 for cytotoxicity by 6-h ^{51}Cr release assay. Target cells were peptide-pulsed TISI cells, an Epstein-Barr virus (EBV)-transformed HLA-A24-positive B cell line, and TISI cells without peptide. Wells were considered positive if the cytotoxicity of the peptide-pulsed TISI cells was 12% higher than that of TISI cells without peptide. All assays were performed in the presence of a 30-fold excess of unlabeled K562 to block non-specific natural-killer (NK) activity. To evaluate the basic immunological status of the patients before treatment, the immune response to flu peptide (RFYIQMCTEL, HLA-A24-

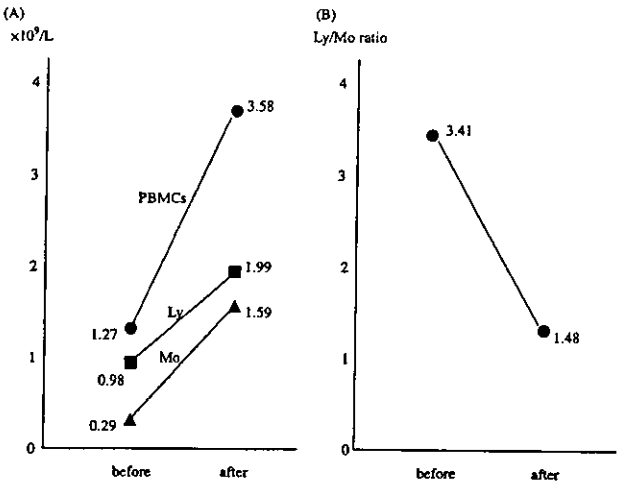

Figure 2. Harvested numbers of PBMCs, lymphocytes (Ly) and monocytes (Mo) by leukapheresis before and after G-CSF mobilization (average numbers derived from 13 patients). A large number of monocyte-rich PBMCs could be mobilized and harvested by leukapheresis after treatment with high-dose G-CSF (A). Consequently, the lymphocyte/monocyte ratio of the harvested PBMCs decreased markedly after G-CSF mobilization (B).

restricted peptide derived from influenza nucleoprotein) was similarly examined to serve as a positive control of recall response.

Results

Patient characteristics. The characteristics of the 18 patients enrolled in this study are summarized in Table I. There were 7 men and 11 women, with a median age of 53 years (range: 28-71). Primary tumors were located in the colon or rectum in 11 patients, the lung in 5 patients, and the stomach in 2 patients. All patients, except 4 with lung cancer, had previously undergone surgical resection of their primary tumors. Unresectable primary or metastatic lesions, diagnosed either at surgery or after surgery, were treated by conventional chemotherapy (5-fluorouracil-based regimens in most cases), radiotherapy, or both. Remaining lesions refractory to these therapies were treated by our study protocol. Such lesions were located in the liver, lung, bone, brain, lymph nodes, peritoneum, or other organs. Seven of the 18 patients had metastatic lesions in more than one organ. All patients had increased serum CEA levels, ranging from 4 to 394 (cut-off value: 2.5 ng/ml). An average period of 7 weeks (range: 4-16 weeks) had elapsed between their last treatment and study entry.

Characteristics of DCs. A large number of mononuclear cells could be mobilized into the peripheral blood after treatment with high-dose G-CSF. The average number of PBMCs obtained by leukapheresis was 3.58×10^9 cells/l, including <1.0% $CD34^+$ hematopoietic stem cells in most cases. Mean lymphocyte/monocyte ratios before and after administration of G-CSF were 3.41 and 1.48, respectively, indicating that the G-CSF-mobilized PBMCs were rich in monocytes (Fig. 2). DCs were generated from the adherent fractions of mobilized PBMCs in the presence of GM-CSF and IL-4. FACS analysis showed that the cells harvested after 7 days' culture consisted of 60% large DCs with high expressions of HLA class I, HLA class II, and CD86; no expression of CD14; and low expressions of CD80 and CD83; and 40% small cells expressing mainly CD3 (data not shown). These results indicated that cells with typical immature DC morphological characteristics and surface phenotypes were successfully generated in all patients in this study.

Clinical outcome and toxicity. All patients received peptide pulsed DC vaccine at least 5 times, and the average number of vaccinations was 12.1 times (range: 5-39) (Table I). None of the patients had major side effects during treatment. However, low-grade toxicity was reported in 2 patients: one (patient F23) had abnormal liver function and leukocytosis; and the other (patient F43) had anemia. These findings were attributed mainly to the rapid progression of cancer rather than to adverse effects of the vaccine. There was no diarrhea or other autoimmune-disease-related gastrointestinal symptoms. We concluded that the vaccine was well tolerated.

The clinical outcomes of the 18 patients are summarized in Table I. No patient showed definite evidence of tumor shrinkage. Despite treatment, many of the patients who had advanced disease with severe immunosuppression showed rapid progression of cancer, associated with elevation of serum CEA levels (normal range: <2.5 ng/ml), and died within <9 months. However, some patients had prolonged stable disease or decreased serum CEA levels. Three of the 5 patients with lung cancer had prolonged and/or marked decreases in serum CEA levels after therapy. Five patients received chemotherapy with new drugs or radiotherapy after the withdrawal of CEA-DC vaccine therapy. The courses of two representative patients who responded clinically to the therapy are described below.

Clinical course of patient F27 (Fig. 3). The patient was a 57-year-old woman with recurrent rectal cancer. The primary tumor in the rectum had been resected in 1996. Multiple lung metastases and right adrenal metastasis, associated with a rise in the serum CEA level, were diagnosed in March 1998. Systemic chemotherapy with a combination of 5-fluorouracil, epirubicine, and mitomycin C was administered, but the patient did not respond, and cancer progressed. CEA-DC vaccine therapy was therefore started in October 1998. The serum CEA level increased transiently after the initiation of therapy, but began to decrease from the beginning of 1999. The serum CEA level remained stable for nearly 1 year. Multiple lung metastases developed very slowly for more than 1 year, and the metastatic lesions in the right adrenal gland showed partial necrotic changes (Fig. 4). CEA652-specific CTL response was confirmed to be enhanced *in vitro* after vaccination. Before vaccination, none of the 48 wells tested showed a peptide-specific CTL response. However, 5 of 40 wells (12.5%) showed a positive peptide-specific CTL response with high peptide-specific cytotoxicity after the eighth vaccination. Moreover, the DTH skin test results became positive after the vaccinations (Table II). However, the serum CEA level began to gradually increase again, and increased spread of the lung metastases and regrowth of the right adrenal metastasis were recognized. We therefore discontinued vaccine therapy after

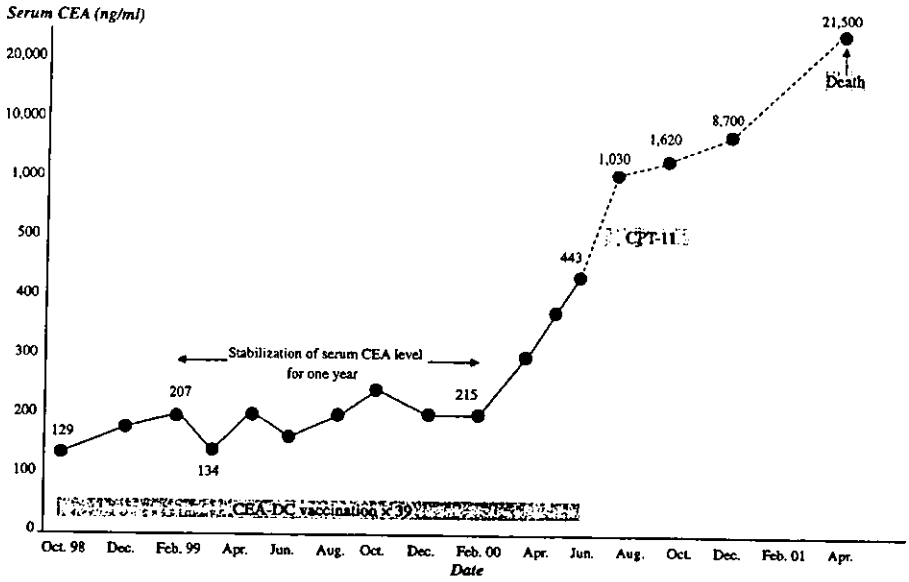

Figure 3. Clinical course of patient F27. A total of 39 CEA-DC vaccinations were administered to this patient. The serum CEA level remained stable for nearly 1 year during the vaccination period.

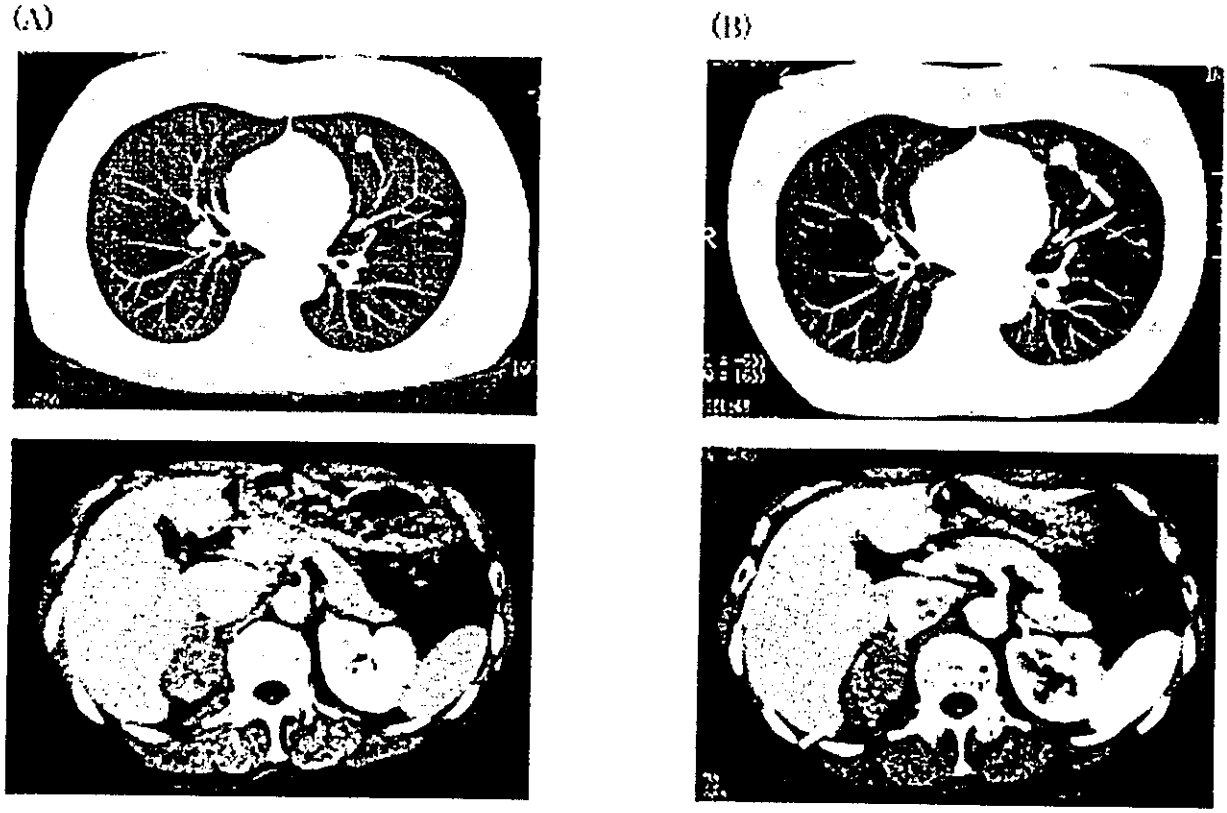

Figure 4. CT scans of patient F27. (A), After the first vaccination (November 5, 1998), bilateral multiple lung metastases and right adrenal metastasis were diagnosed. (B), After the 12th vaccination (April 24, 1999), the size of the metastatic lesions in the left lung increased slightly (white arrow), but the number of lung metastases did not increase, and partial necrotic change was confirmed in the right adrenal gland metastasis (white arrow).

39 times and gave the patient CPT-11-based chemotherapy. She died of cancer 31 months after the initiation of vaccine therapy. Multiple hepatic metastases were recognized when she died.

Clinical course of patient F37 (Fig. 5). The patient was a 52-year-old woman with recurrent lung cancer. The primary tumor in the lower lobe of the left lung had been resected in July 1995. Metastasis to the sacral bone, associated with a

Table II. Immunological response to CEA-DC vaccine therapy.

Patient no.	Age/Sex	*In vitro* CTL response			DTH		Serum CEA
		Flu (pre)[a]	CEA (pre)	CEA (post)[b]	pre	post	
F23 (rectal carcinoma)	54/F	-	-	-	-	-	↑
F32 (rectal carcinoma)	56/M	-	-	-	-	-	↑
F38 (colon carcinoma)	28/F	+	+	+	-	-	↑
F42 (colon carcinoma)	61/F	-	-	-	-	-	↑
F43 (colon carcinoma)	64/F	+	+	NT	-	-	↑
F44 (lung carcinoma)	69/M	-	-	-	-	-	↑
F40 (colon carcinoma)	42/F	-	-	-	-	-	→↑
F41 (gastric carcinoma)	33/F	+	+	+	-	-	→
F27 (rectal carcinoma)	57/F	+	-	+	-	+	→
F37 (lung carcinoma)	52/F	+	+	+	-	+	↓
F101 (lung carcinoma)	39/M	+	+	+	-	+	↓

[a]pre, before CEA-DC vaccination. [b]post, after CEA-DC vaccination. NT, not tested.

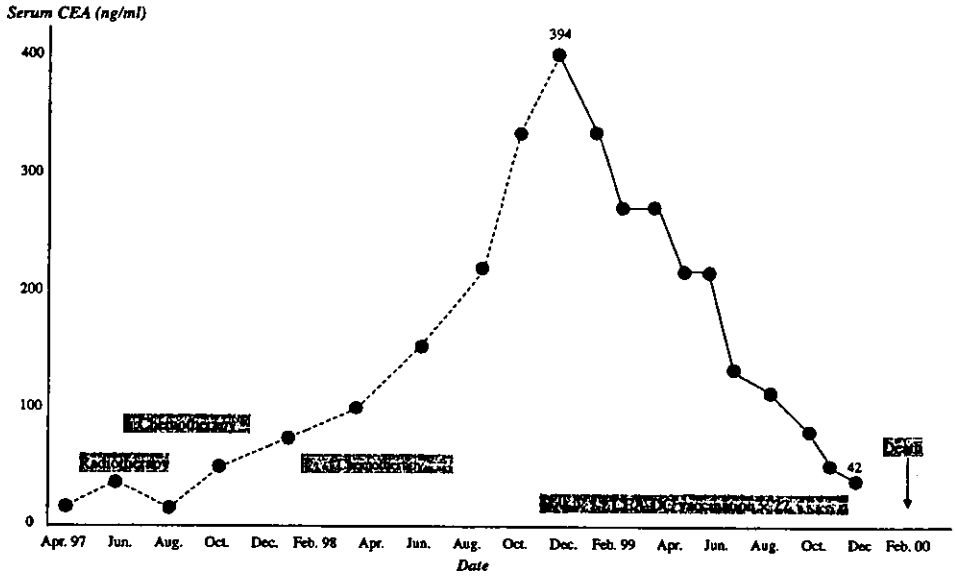

Figure 5. Clinical course of patient F37. During CEA-DC vaccine therapy, there was a marked, long-term decrease in the serum CEA level (before vaccination, 394 ng/ml; after 22nd vaccination, 42 ng/ml). However, the patient died of progressive cancer in February 2000.

rise in the serum CEA level, was diagnosed in April 1997. She received radiotherapy and chemotherapy (paclitaxel, carboplatin). However, cancer could not be controlled, and the serum CEA level increased markedly. The patient was enrolled in our study in December 1998. Vaccination was administered a total of 22 times from December 1998 through December 1999. Treatment was associated with a prolonged decrease in the serum CEA level from 394 to 42 ng/ml. This marked decease was thought to indicate disease regression. However, an MRI scan obtained after 17 vaccinations revealed distinct progression of the sacral lesion. In addition, a solitary brain metastasis appeared 6 months after the initiation of CEA-DC vaccine therapy (Fig. 6). The patients' serum CEA level thus did not reflect tumor progression. She died of cancer 14 months after the initiation of therapy. Immunohistochemical analysis of the resected primary lung adenocarcinoma tissue and punched-out metastatic sacral bone tissue obtained when she died revealed strong staining with monoclonal antibody against CEA in both tissues (data not shown). In this patient, the DTH skin test turned positive after the vaccinations, but there was no enhancement of CEA652-specific CTL response *in vitro* (Table II, before: 3/32 wells, after: 1/24 wells).

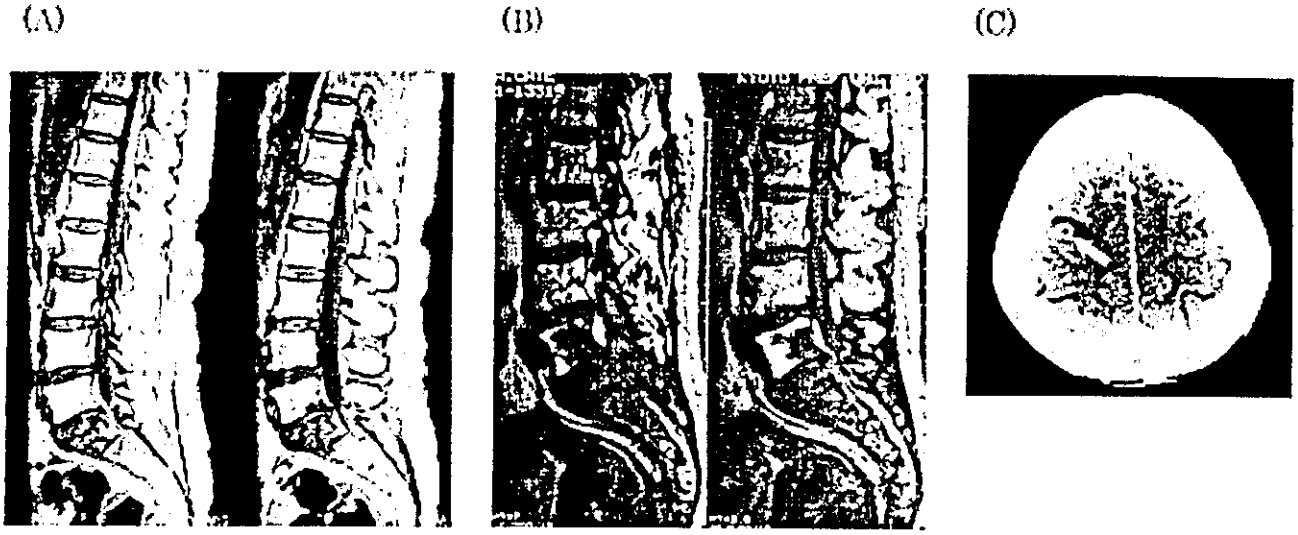

Figure 6. MRI and CT scans of patient F37. (A), After the eighth vaccination (April 20, 1999), metastatic sacral lesions were almost localized to the S1 segment. (B), After the 17th vaccination (September 29, 1999), lesions spread throughout the entire sacral bone and invaded the lower lumbar vertebra. (C), After the 19th vaccination (October 29, 1999), a solitary brain metastasis appeared (white arrow).

Immunological response. Both the DTH response and the peptide-specific *in vitro* CTL response were examined in 11 patients. The results are shown in Table II. Clinically, 4 of the 11 patients had decreased or stable serum CEA levels for >3 months in response to vaccine therapy.

DTH responses to CEA652-pulsed DCs were negative in all patients before vaccination. However, 3 patients (F27, F37, F101) showed strong positive responses with skin erythema >20 mm in diameter after 5 vaccinations. These 3 patients had decreased or stable serum CEA levels during vaccine therapy. Some other patients showed faint erythema measuring <5 mm, but these reactions were judged to be clinically insignificant.

CEA652-specific CTL responses either before vaccination or after at least 5 vaccinations were positive in 6 of 11 patients. Four of the 6 patients with positive responses had decreased or stable serum CEA levels during vaccine therapy (F41, F27, F37, F101). Moreover, 5 of these 6 patients also showed positive *in vitro* CTL responses to flu-peptide. CEA652-specific CTL response was enhanced after vaccination in 2 patients (F27-before: 0/48 wells, after: 5/40 wells, F101-before: 2/48 wells, after: 4/48 wells).

Discussion

DCs, antigen-presenting cells capable of priming naïve T cells to specific antigens in an HLA-restricted manner, induce potent antitumor immunity *in vitro* and *in vivo* (6). In humans, immature DCs can easily be induced *in vitro* from non-proliferating $CD14^+$ monocytes in peripheral blood by treatment with GM-CSF and IL-4 (4). Clinical trials of active immunotherapy with monocyte-derived DCs have been conducted since the latter half of the 1990s. Many types of monocyte-derived DCs have been tried, and the most promising results have been obtained in the more immunogenic tumors, such as malignant melanoma, renal cell carcinoma, and lymphoma (7,8). Studies of other refractory malignancies, such as gastrointestinal or lung cancer, are relatively scant (9,10). We therefore conducted a pilot study of DC-based immunotherapy in patients with gastrointestinal or lung cancer, two major causes of cancer-related mortality in Japan and other countries. As a tumor antigen for DC loading, we used a CTL epitope peptide, CEA652. $CD8^+$ CTLs recognize this 9-mer peptide presented by MHC class I molecules on the cell surface (3). Although use of a defined peptide for DC loading has several intrinsic disadvantages as compared with other types of antigens, this techniques offers many clinical advantages, including no need for tumor tissues or cells, easy control of input materials, and more accurate monitoring of the induced immune response (11). We therefore used a defined peptide. Because CEA is expressed not only in gastrointestinal and lung epithelial tumor cells but also weakly in normal colonic mucosa, autoimmune adverse effects had to be carefully monitored (12). None of our patients had any autoimmune reactions such as diarrhea, consistent with the results of other clinical trials of immunotherapy targeting CEA (9,10). Experimental studies of murine tumor models have also confirmed the absence of autoimmunity (13,14). We therefore concluded that CEA, an overexpressed self antigen, is a safe target molecule for tumor-specific immunotherapy.

Besides confirming safety and feasibility, one of the major aims of our study was to determine whether a vaccine consisting of DCs pulsed with CEA652 is clinically active. To permit frequent and repeated treatment, we used monocytes mobilized with G-CSF as a cell source of DCs. The leukapheresis products after G-CSF-mobilization were most abundant in monocytes; consequently, the yield of DCs was 5.5-fold more than that without mobilization (Fig. 2). The allo-stimulatory activity of DCs generated from G-CSF-mobilized monocytes was at least as strong as that of DCs generated from non-mobilized monocytes (data not shown). Choi *et al* also reported the efficacy and advantages of DC

induction from G-CSF mobilized monocytes (15). By cryopreserving mobilized monocytes obtained by a single session of leukapheresis, we could produce a large number of clinically usable DCs.

As for tumor shrinkage, no patient showed objective evidence of clinical response. However, the 4 patients who had prolonged stable disease (patient F27) or decreased/stable serum CEA levels (patients F27, F41, F37, F101) were considered to have clinically responded to therapy. All 4 of these patients showed positive *in vitro* CTL responses both to a recall antigen (flu-peptide) before treatment and to CEA652 after treatment (Table II). The preservation of basic immunological status is thus considered very important for a response to cancer vaccine therapy. Moreover, 3 of these 4 patients showed a positive DTH response after treatment (Table II), whereas none had a positive response before treatment. The DTH response reflects the development of systemic cell-mediated immunity and may be important for immunological monitoring after class I-restricted peptide immunization (9,16,17). Our results suggest that the DTH response also might be a useful surrogate marker of clinical response to cancer vaccine therapy.

Five patients in our study received chemotherapy with new agents after the withdrawal of vaccine therapy. All of these patients had mildly or moderately advanced disease and were in good physical condition. Consequently, survival after the initiation of vaccine therapy was relatively prolonged [mean, 26.8 months (data not shown)]. Patients F27 and F48 showed responses typical of this subgroup of patients. These findings suggest that cancer vaccine therapy, which stimulates patients' antitumor immunity through the induction or enhancement of T-cell immunity, would be most beneficial in patients with relatively early cancer who have not yet received immunosuppressive chemotherapy. Clearer therapeutic guidelines may improve the outcome of advanced cancer, but the response to cancer vaccine therapy must be confirmed in larger controlled studies.

We described the clinical courses of patients F27 and F37, two examples of patients who apparently responded to vaccine therapy. In patient F27, long-term tumor dormancy was ascribed to *in vivo* CEA-specific CTL induction after vaccine therapy (18). In case F37, serum CEA levels did not parallel tumor progression, and the patient died of cancer despite markedly decreased serum CEA levels after 22 vaccinations. This was considered a very rare phenomenon. Perhaps use of a single, defined CEA-derived CTL epitope led to the immunological eradication of CEA-positive tumor cells by CEA-specific CTLs, and the tumor escaped immune recognition. However, immunohistochemical analysis showed that the metastatic sacral bone tissue obtained when she died was as strongly CEA-positive as the resected primary lung cancer tissue (data not shown). Previous studies in patients with colorectal cancer have shown no correlation between the level of CEA in sera and the expression level of CEA in tumors (19). This rare disassociation between serum CEA levels and tumor progression is very difficult to explain, but may be attributable to some type of immunological escape occurring after specific immunotherapy targeting a single molecule.

Several clinical or experimental studies have suggested ways to improve CEA-targeted immunotherapy. One way involves the use of altered peptide ligands (APLs). CAP-1-6D, an APL of CEA-derived, HLA-A2.1-restricted CTL epitope, CAP-1 ($CEA_{605-613}$), has been identified. In CAP-1-6D, aspartate has been substituted for asparagine at position 610 (610D). CAP-1-6D possesses increased potency in inducing CTLs against CEA (20,21). Fong *et al* (10) demonstrated that immunization with Flt3 ligand-expanded DCs loaded with CAP-1-6D could lead to CEA-specific immunity and clinical response. They reported on 2 patients with metastatic colorectal cancer who had complete tumor regression (10). Another way to improve CEA-targeted immunotherapy involves the identification of an antigenic epitope for helper T lymphocytes from CEA. A newly identified 15-mer helper T-cell epitope from CEA, a peptide of CEA occupying residue positions 653 to 667, $CEA_{653-667}$, is effective for inducing *in vitro* T helper responses in the context of the HLA-DR4, -DR7, and -DR9 alleles (22). Fortunately, this helper T-cell epitope overlaps with the HLA-A24-restricted CTL epitope, CEA652, used in the present study. Because HLA-A24, -DR4, and -DR9 are common MHC alleles in the Japanese population, use of a relatively small peptide, such as $CEA_{652-667}$, could be a new and effective strategy for the immunotherapy of CEA-expressing solid cancers in Japan. DC-based immunotherapy with CEA-derived, HLA-A24-restricted peptides may be further refined by identifying potent APLs of CEA652 and by concurrent adjuvant therapy with commercially available immunomodulatory agents that have antitumor activity (23).

In conclusion, active specific immunotherapy using DCs pulsed with CEA652 is a safe and feasible treatment procedure that holds promise of being clinically effective in selected cases of metastatic gastrointestinal or lung adenocarcinomas. Our results will hopefully encourage the further development of DC-based immunotherapy with CEA-derived peptides and promote their use for the management of refractory solid cancers that express CEA.

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Mobilization of peripheral blood stem cells (PBSCs) after etoposide, adriamycin and cisplatin therapy, and a multimodal cell therapy approach with PBSCs in advanced gastric cancer

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Abstract. The EAP combination of etoposide (ETP), doxorubicin (ADM) and cisplatin (CDDP) has been reported to be highly active for advanced gastric cancer. However, it is associated with severe myelotoxicity, and its use has declined. We examined whether peripheral blood stem cells (PBSCs) could be mobilized during hematopoietic recovery after EAP, and assessed the possibility of using multimodal cell therapy with PBSCs for the treatment of advanced gastric cancer. Five men with advanced gastric adenocarcinoma were enrolled. All patients were chemotherapy-naïve. EAP (ETP, 360 mg/m^2; ADM, 40 mg/m^2; CDDP, 80 mg/m^2) was given to each patient, and myelotoxicity was carefully monitored. Granulocyte colony-stimulating factor was administered after the neutrophil nadir, and PBSCs were collected by leukapheresis during hematopoietic recovery. The median nadir of the neutrophil count after EAP was 225/ml, occurring between day 17 and 20. Sufficient numbers of PBSCs [CD34(+) cells, CFU-GM] could be mobilized in 4/5 patients. A 45-year-old patient with extended lymph node metastasis received high-dose EAP with peripheral blood stem cell transplantation (PBSCT), followed by cancer vaccine therapy with dendritic cells (DCs), induced from cryopreserved PBSCs.Both high-dose EAP with PBSCT and DC-based immunotherapy was safely performed for the first time against gastric cancer. Although associated with severe myelotoxicity, EAP can mobilize sufficient numbers of PBSCs during hematopoietic recovery. Multimodal cell therapy combining high-dose chemotherapy with PBSCT and DC-based immunotherapy is feasible and can be a reasonable approach in advanced gastric cancer.

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Key words: gastric cancer, high-dose chemotherapy, peripheral blood stem cell transplantation, dendritic cell, cancer vaccine therapy

Introduction

A combination of etoposide (ETP), doxorubicin (Adriamycin, ADM), and cisplatin (CDDP) (EAP) was firstly reported by Preusser *et al* (1) to be highly active against locally advanced or metastatic gastric cancer (2,3). The effectiveness of this regimen has been disputed by some (4,5), but confirmed by others, with many reporting clinical response rates of approximately >50% (6,7). A major drawback of EAP is a high frequency of severe myelotoxicity, especially grade 3 or 4 leukopenia and neutropenia, which has lead to a number of deaths (4,5,8). Despite the development of modified regimens for EAP (6), the persistence of severe, life-threatening myelotoxicity has led to a recent decline in its use.

On the other hand, an important advantage of EAP, which does not include 5-fluorouracil (5-FU), the drug most commonly used to treat gastric cancer, is the ability to escalate the doses of individual agents in the regimen, provided that bone marrow failure does not develop. Because myelotoxicity is generally the only major side effect of ETP, dose intensity can be increased by administering appropriate adjuvant therapy to rescue bone marrow, potentially resulting in a better response. Dose escalation of ETP has been confirmed to be effective against many other solid tumors (9). Some investigators have given high-dose chemotherapy (HDC) with EAP to patients with advanced gastric cancer who concurrently received cytokine support or hematopoietic stem cell transplantation. Ajani *et al* (10) described 26 patients given high-dose EAP with granulocyte/macrophage colony-stimulating factor (GM-CSF) support. Although this regimen was very active against locally advanced disease, it caused substantial toxicity and 2 deaths (10). Subsequently, Suzuki

et al reported that 8/10 patients with late-stage gastric cancer had a partial response (PR) to high-dose EAP with autologous bone marrow transplantation (BMT), without severe complications (11). More recently, rapid progress in peripheral blood stem cell transplantation (PBSCT) (12) led to 2 clinical trials of HDC with autologous PBSCT for advanced gastric cancer, one reported by Berdel *et al* in 1996 and 2001 (13,14), and the other by Reichle *et al* in 2003 (15). In both studies, EAP was used as induction therapy to mobilize peripheral blood stem cells (PBSCs) and was followed by a high-dose MCEB regimen [a combination of mitomycin C (MMC), CDDP, ETP and carmustine (BCNU)] plus PBSCT, with or without surgery. No HDC-related death was reported in any of the 31 patients who received PBSCT in these studies, and prolonged disease-free survival was achieved in some patients with locally advanced disease who responded to treatment plus surgery (14,15). Both of these studies focused on the anti-tumor effect of HDC. Consequently, although PBSCs were mobilized after EAP in 34 patients, neither study provided details on the technique used to mobilize stem cells or discussed the relation between the extent of bone marrow suppression and the efficiency of stem cell mobilization.

In this study, we gave a regular dose of EAP to 5 patients with primary advanced gastric cancer and harvested PBSCs during hematopoietic recovery after EAP. The course of bone marrow suppression after EAP and the efficiency of the resultant mobilization of stem cells were examined in all 5 patients. In addition, 2 types of multimodal cell therapies using PBSC were given to 1 patient with extended lymph node metastases. The first was HDC with PBSCT, and the second was active specific immunotherapy with dendritic cells (DCs) induced *in vitro* from monocytes in PBSCs.

To our knowledge, this is the first report to describe a multimodal cell therapy approach with PBSCs in advanced gastric cancer. In addition to reviewing the current status of HDC with hematopoietic stem cell transplantation for advanced gastric cancer, we discuss future directions for multimodal cell therapy.

Patients and methods

Patients. Five patients with metastatic gastric adenocarcinoma were enrolled. All patients had histologically proven primary disease, with measurable or assessable lesions. Our ethics committee approved the study protocol, and all patients gave informed consent before enrollment. The protocol required that all patients (i), were between 18 and 75 years of age; (ii), had an Eastern Cooperative Oncology Group performance status (PS) <3; (iii), had adequate cardiac, pulmonary, hepatic, renal and hematologic function [peripheral blood white blood cell count (WBC) >3,000 /μl, peripheral blood platelet count (PLT) >100,000 /μl]; and (iv), had no prior chemotherapy, radiotherapy or gastric resection. Patients were excluded if they (i), had infectious disease; (ii), were receiving steroid therapy; or (iii), were pregnant. All patients were hospitalized for treatment.

Biopsy specimens of the primary lesions were histologically classified into 2 groups. The first comprised differentiated-type cancer, including papillary adenocarcinoma and well- and moderately-differentiated tubular adenocarcinoma, roughly consistent with intestinal-type cancer according to

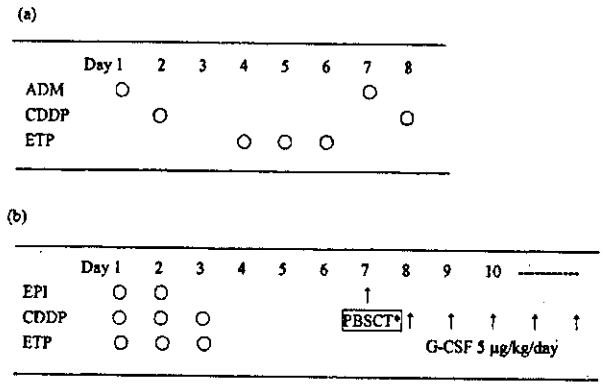

Figure 1. (a), Original EAP regimen: ADM, adriamycin 20 mg/m² x 2; CDDP, cisplatin 40 mg/m² x 2; ETP, etoposide 120 mg/m² x 3 (100 mg/m² in patients >60 years old). (b), High-dose EAP regimen rescued by PBSCT: EPI, epirubicin 40 mg/m² x 2; CDDP, cisplatin 40 mg/m² x 3; ETP, etoposide 200 mg/m² x 3. *Autologous peripheral blood stem cell transplantation was performed with retransfusion of 3 x 10⁶ CD34(+) cells/kg body weight of the recipient, day 7. G-CSF, granulocyte colony-stimulating factor.

Lauren's classification (16). The other group consisted of undifferentiated-type cancer, including poorly differentiated adenocarcinoma and signet-ring cell carcinoma, which were identical to diffuse-type cancer (16). The gross findings of each primary lesion were evaluated according to the 13th Edition of Japanese Classification of Gastric Carcinoma, and disease was staged according to the 5th Edition of UICC-TNM classification (ICD-O C16).

Chemotherapy regimen (original EAP). EAP was administered as originally described by Preusser *et al* (1), and consisted of: intravenous (IV) ADM 20 mg/m² on days 1 and 7; IV CDDP 40 mg/m² on days 2 and 8; and IV ETP 120 mg/m² on days 4-6. The daily dose of ETP was decreased to 100 mg/m² in patients >60 years (Fig. 1a). ADM was given by 20-min short infusion, ETP by 1-h infusion in 500 ml 0.9% saline, and CDDP by 2-h infusion in 500 ml 0.9% saline with anti-emetic drugs and with adequate hydration before and after treatment. During and after EAP therapy, complete blood counts and serum chemical analyses were frequently performed.

Mobilization and harvest of PBSCs. A dose of 5 μg/kg of glycosylated recombinant human granulocyte colony-stimulating factor [G-CSF (lenograstim); Neutrogin®, Chugai, Tokyo, Japan] was given to each patient subcutaneously once daily, from the day of the nadir of the neutrophil count after the first cycle of EAP. After WBC recovered to 5,000 /μl, leukapheresis collections of PBSCs were carried out for 2 consecutive days using a CS3000 cell separator (Baxter Limited, Deerfields, IL, USA). A total volume of 15-20 l of blood was processed in each patient. A small percentage of true hematopoietic stem cells were included in the total leukapheresis product (bulk-PBSCs) as mononuclear cells. They were cryopreserved in liquid nitrogen after enumeration by flow cytometry (Fig. 2).

Enumeration of PBSCs. Hematopoietic stem cells in harvested mononuclear cells were enumerated by 2 different assays, flow cytometry and hematopoietic colony formation assay.

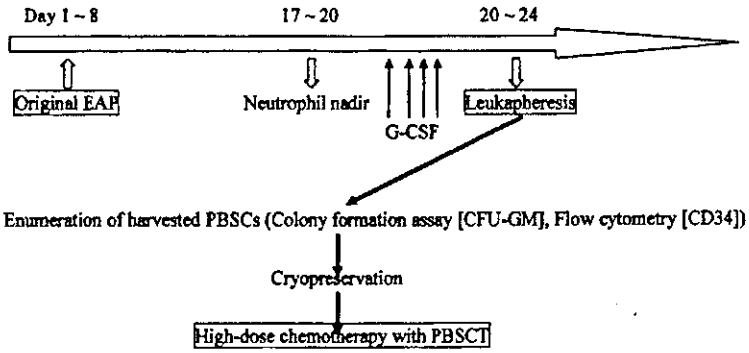

Figure 2. Mobilization and harvest of PBSCs after the original EAP. G-CSF, granulocyte colony-stimulating factor; CFU-GM, granulocyte/macrophage colony forming unit; PBSCT, peripheral blood stem cell transplantation.

Percentages of CD34(+) cells in harvested mononuclear cells were analyzed by flow cytometry using FACS Calibur (Becton Dickinson, San Jose, CA, USA) and CellQuest software on the days of leukapheresis. Immunofluorescent staining of mononuclear cells was performed with FITC-conjugated anti-CD34 (Becton Dickinson). Colony formation assay was carried out as described by Sonoda *et al* (17). Samples of cryopreserved mononuclear cells were thawed about 1 week after harvest and cultured in medium containing G-CSF, GM-CSF, stem cell factor (SCF), erythropoietin (Epo) and interleukin-3 (IL-3) for 2 weeks, and all colonies were scored *in situ* on an inverted microscope.

High-dose chemotherapy with PBSCT. HDC rescued by PBSCT was tried in 1 patient (Patient 5). The chemotherapy consisted of IV epirubicin (EPI, epi-adriamycin) 40 mg/m^2 on days 1 and 2, IV CDDP 40 mg/m^2 on days 1-3, and IV ETP 200 mg/m^2 on days 1- 3 (high-dose EA'P therapy) (Fig. 1b). EPI was used instead of ADM to reduce cardiotoxicity. Each drug was given in the same manner as described for the original EAP protocol. PBSCT was carried out on day 7. Bulk-PBSCs including 3x10^6 CD34$^+$ cells/kg body weight were thawed rapidly and infused immediately. During and after HDC rescued by PBSCT, complete blood counts and serum chemical analyses were frequently performed. imipenem and cilastatin (500 mg) were administered twice a day from day 7 until WBC was >2,000/ μl, and gamma globulin (2.5 g) was administered twice a day from day 7 for 3 days. G-CSF (5 μg/kg) was given subcutaneously from day 8 until WBC was >5,000/ μl. When the PLT was <30,000 /μl, 10 units of irradiated (15 Gy) platelet were infused. Nutrition support was provided by intravenous hyperalimentation.

In vitro induction of DCs from monocytes in bulk-PBSCs, and DC-based cancer vaccine therapy with CEA-derived CTL epitope. Cancer vaccine therapy with the use of DCs and CEA-derived, HLA-A24-restricted 9-mer CTL epitope peptide [CEA652 (TYACFVSNL), Takara, Shiga, Japan] (18) was tried in 1 patient (Patient 5). The study protocol was approved by the Institutional Ethics Review Committee, Kyoto Prefectural University of Medicine in October, 1998, and written informed consent was obtained from the patient (19,20). The peptide was confirmed to be >95% pure and endotoxin-free. Peptide-pulsed DCs were generated from monocytes in cryopreserved bulk-PBSCs according to our previously described method (19,20). Bulk-PBSCs (10^9 cells) were thawed rapidly, suspended in 50 ml of complete medium [RPMI1640 (Nikken, Kyoto, Japan) supplemented with 5% heat-inactivated autologous serum and gentamycin (Fujisawa Pharmaceutical, Osaka, Japan)], and plated in 5 225 cm^2 cell culture flasks. The flasks were incubated in a 5% CO_2 incubator at 37°C for 2 h, and non-adherent cells were removed. Adherent cells (monocyte-enriched fraction) were cultured in 50 ml of complete medium containing 1,000 U/ml each of GM-CSF (Kirin, Tokyo, Japan) and recombinant human interleukin-4 (IL-4; Genzyme, Minneapolis, MN, USA). After 7 days of culture, DCs were harvested, washed twice and suspended in 3 ml of phosphate-buffer saline (PBS), containing 1% human albumin (Fujisawa). Phenotypic analyses of DCs were performed with FACS Calibur and CellQuest software. Immunofluorescent staining was done with the following monoclonal antibodies (mAbs): FITC-conjugated anti-CD14, PE-conjugated anti-CD80 (Becton Dickinson), anti-CD83 (Immunotech, France) and anti-CD86 (Ancell, Bayport, MN, USA). DC preparations were confirmed to be endotoxin-free (<10 pg/ml in the supernatant). CEA652 peptide was dissolved at 40 μg/ml and added to the DC suspension at room temperature. After 4 h, the cells were washed, prepared as a cell suspension in 3 ml of saline containing 1% human albumin, and used as the vaccine (CEA-DC vaccine). This vaccine, which included approximately 5x10^7 DCs, was injected intra-dermally and subcutaneously at the same site in the cervical and axillary regions via a 26-G needle. Continuous IV administration of 720,000 U/day interleukin-2 (teceleukin; Imuneas®, Shionogi, Osaka, Japan), immediately followed the vaccine therapy for 3 consecutive days. After 2 weeks, the same treatment was repeated once.

To monitor delayed-type hypersensitivity response, skin tests were performed before and after the vaccine therapy. CEA652 peptide (10 μg) in 0.1 ml saline was injected intradermally in the antebrachial region. As a control, the same volume of saline without CEA652 was tested simultaneously. A positive skin-test reaction was defined as skin erythema >5 mm in diameter, 24 h after injection.

Toxicity and response evaluation. All toxic reactions were graded according to the National Cancer Institute (NCI) common toxicity criteria, version 2.0. Tumor size was

Table I. Patient characteristics and clinical response to EAP.

Patient no.	Age/Sex	PS	Gross findings of primary lesions[a]	Histology	Cancer spread	Response to original EAP
1	55/M	1	Type 5	Differentiated	Virchow and paraaortic LN meta., bone meta.	PR: disappearance of Virchow meta., regression of paraaortic LN meta.
2	57/M	2	Type 3	Undifferentiated	Direct invasion to the pancreas, bone meta.	PR: disappearance of direct invasion to the pancreas, regression of bone meta.
3	72/M	1	Type 4	Undifferentiated	Direct invasion to the pancreas and transverse colon, peritoneal dissemination	NC
4	39/M	0	Type 4	Undifferentiated	Paraaortic LN meta., peritoneal dissemination	NC
5	45/M	0	Type 3	Differentiated	Esophageal invasion, paraaortic LN meta.	PR: Regression of primary lesion and paraaortic LN meta.

PS, ECOG (Eastern Cooperative Oncology Group) performance status; M, male; LN, lymph node; meta., metastasis; PR, partial response; CR, complete response. [a]Gross findings of primary lesions were evaluated according to the 13th Japanese Classification of Gastric Carcinoma.

Table II. Neutrophil nadir and harvested PBSCs [$CD34^+$ cells, CFU-GM] after the original EAP.

Patient no.	Neutrophil nadir	Harvested mononuclear cell number by leukapheresis	% of $CD34^+$ cells in harvested mononuclear cells	Harvested $CD34^+$ cell number	Harvested CFU-GM
1	323 /μl	2.0×10^{10} (Day 20)	1.33	5.3×10^6 /kg[a]	7.7×10^5 /kg
	(Day 17)	1.8×10^{10} (Day 21)	2.10	7.6×10^6 /kg	NT
2	29 /μl	1.9×10^{10} (Day 21)	2.57	8.8×10^6 /kg	5.0×10^5 /kg
	(Day 17)	1.8×10^{10} (Day 22)	2.10	6.8×10^6 /kg	11.2×10^5 /kg
3	0	1.3×10^{10} (Day 22)	5.78	17.9×10^6 /kg	20.0×10^5 /kg
		1.2×10^{10} (Day 23)	7.24	20.7×10^6 /kg	28.8×10^5 /kg
4	578 /μl	(Leukapheresis was not performed)			
	(Day 20)				
5	192 /μl	1.6×10^{10} (Day 22)	2.16	6.0×10^6 /kg	11.6×10^5 /kg
		1.8×10^{10} (Day 23)	3.30	10.2×10^6 /kg	9.0×10^5 /kg

PBSCs, peripheral blood stem cells; CFU-GM, granulocyte/macrophage colony-forming units; NT, not tested. [a]Harvested $CD34^+$ cell numbers and CFU-GM are indicated per kg body weight.

determined before initiation of therapy and every 4 weeks after initiation of therapy, mainly by computed tomography (CT). The World Health Organization (WHO) criteria were used to define treatment response.

Results

Patient characteristics and clinical responses to EAP. The patients characteristics and clinical responses to EAP are summarized in Table I. All 5 patients were male, with a median age of 54 years (range, 39-72). Two patients had differentiated-type adenocarcinoma, and 3 had undifferentiated-type adenocarcinoma. All patients had T3 or T4 primary tumors with metastatic lesions (Stage IV). One patient had Virchow metastasis, 3 had lymph node metastases around the abdominal aorta, 2 had bone metastases and 2 peritoneal dissemination.

One patient (Patient 5) received 2 courses of EAP separated by a 5-week interval, and all others received 1 course of EAP. Three patients had a partial response (PR), and 2 had no change (NC) after EAP.

Myelotoxicity of EAP. The main toxicity of EAP is myelotoxicity. Four patients (80%) had grade 4 leukopenia and neutropenia, and 1 had grade 3 leukopenia and neutropenia. The median nadir of the neutrophil count was 225 /μl (range, 0-578), occurring between day 17 and 20 (median, day 18)

Table III. Subpopulation of bulk-PBSCs harvested by leukapheresis[a].

	Percentage of cells (%)
Lymphocytes	52.0
Monocytes	37.0
Atypical lymphocytes	2.0
Metamyelocytes	1.0
Promyelocytes	4.0
Blast cells[b]	3.0

[a]Subpopulation of the 2nd leukapheresis product in Patient 5 is shown as an example. [b]Blast cells are nearly equivalent to CD34$^+$ cells.

(Table II). Severe but manageable infection (central venous catheter-related infection) occurred in 1 patient. Grade 3 thrombocytopenia requiring platelet transfusion developed in 1 patient. Non-hematologic toxicity was mild, except for grade 3 nausea and vomiting refractory to anti-emetic drugs in 1 patient.

PBSCs mobilized and harvested after EAP. Four patients underwent leukapheresis to harvest PBSCs. The other patient (Patient 4) did not undergo leukapheresis because myelosuppression severe enough to mobilize PBSCs did not develop, and CD34$^+$ cells were not detected in peripheral blood on flow cytometry during hematopoietic recovery. Leukapheresis was carried out from days 20-24, after WBC had recovered to 5,000/μl following administration of G-CSF. Patients received 2 sessions of leukapheresis, given on 2 consecutive days. The median number of harvested mononuclear cells was $1.7x10^{10}$ (range, $1.2\text{-}2.0x10^{10}$). The median percentage of CD34$^+$ cells included in mononuclear cells harvested by each session of leukapheresis was 3.32 (range, 1.33-7.24), and the median number of harvested CD34$^+$ cells in each leukapheresis product was $10.4x10^6$ (range, $5.3\text{-}20.7x10^6$) /kg body weight (Table II). A representative subpopulation of the harvested mononuclear cells (bulk-PBSCs) is shown in Table III. Apart from CD34$^+$ cells, the bulk-PBSCs included primarily lymphocytes and monocytes. Colony formation assay showed that a median number of $13.3x10^5$ (range, $5.0\text{-}28.8x10^5$) granulocyte/macrophage colony-forming units (CFU-GM)/kg body weight were included in each leukapheresis product.

Clinical course and multimodal cell therapies with PBSCs in Patient 5: a case report. We applied a multimodal cell-therapy approach with PBSCs after administering 2 courses of the original EAP regimen to a patient with extensive lymph node metastases (Patient 5). The clinical course of the patient is described below and summarized in Fig. 4.

A 45 year-old man was referred for further examination of dysphagia and a markedly elevated serum CEA level (2,400 ng/ml; cut off value, 2.5 ng/ml) in October, 1999. An upper gastrointestinal series and endoscopic examination revealed a large type 3 advanced gastric cancer, arising in the cardia and invading the lower esophagus. Examination of a biopsy specimen showed differentiated-type adenocarcinoma. Abdominal CT scans showed extensive lymph node involvement around the stomach, abdominal aorta, and both iliac arteries. There was no evidence of distant metastasis. The diagnosis was T3, N3, M0, Stage IV advanced gastric cancer. The patient was given 2 courses of pre-operative chemotherapy with the original EAP regimen. Chemotherapy resulted in partial regression (PR) of the primary lesion and remarkable regression of the lymph node metastases. No clinically significant lymph node swelling was detected on abdominal CT scans 4 weeks after the second course of EAP. Leukapheresis was carried out after the first course of EAP, and sufficient numbers of PBSCs were harvested and cryopreserved (Table II). Total gastrectomy with lower esophagectomy, splenectomy and D2 lymph node dissection was performed in February, 2000. Biopsy specimens of lymph nodes around the abdominal aorta, obtained at operation, showed no viable cancer cells. However, some lymph node metastases were found around the stomach (pN1). The serum CEA level fell to 46 ng/ml after surgery, but remained above the upper limit of normal. We had planned to administer HDC with PBSCT immediately after recovery from surgery, but this treatment was not feasible because of a minor leakage from the esophagojejunostomy. The patient wanted to be discharged from the hospital and to continue therapy on an outpatient basis. Therefore, we administered S-1 (TS-1®, Taiho, Tokyo, Japan), an oral dihydropyrimidine dehydrogenase-inhibitory fluoropyrimidine. Two courses of S-1 (each course consisting of 4

Day	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
WBC /μl				8,600	5,100	3,900	2,600	2,800	1,200	800	900	1,100	600	500	2,300	8,200	23,500	18,500
Neut. /μl				6,700	3,490	2,680	1,530	1,890	150	10	10	10	0	90	890	5,560	19,000	15,200
RBC x 10^6/μl				287	317	317	304	298	287	262	255	262	251	215	230	237	242	230
Hb g/dl				10.2	11.2	11.2	10.7	10.6	10.2	9.3	9.0	9.3	9.0	7.7	8.3	8.8	8.5	8.4
Plt. x 10^4/μl				20.8	17.6	15.5	12.6	10.4	8.7	5.2	2.8	5.9	2.9	3.9	2.9	3.8	4.0	10.5
G-CSF (5 μg/kg/day):				○	○	○	○	○	○	○	○	○	○	○	○			
Plt. transfusion (10U):				○					○				○					

Figure 3. Hematopoietic recovery after high-dose EA'P rescued by PBSCT (Patient 5). PBSCT, peripheral blood stem cell transplantation; Plt., platelets; G-CSF, granulocyte colony-stimulating factor.

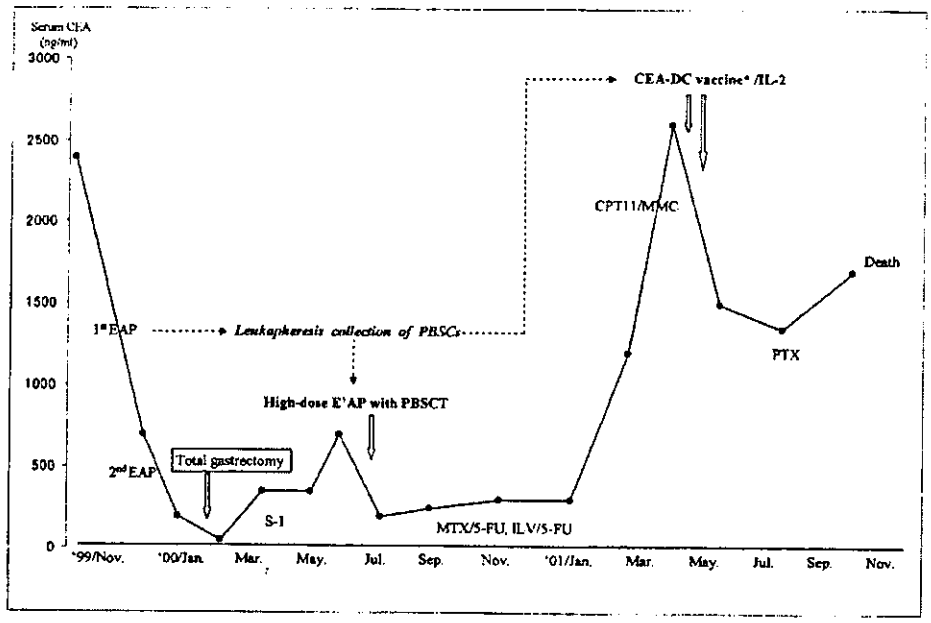

Figure 4. Clinical course (Patient 5). PBSCT, peripheral blood stem cell transplantation; MTX, methotrexate; 5-FU, 5-fluorouracil; ILV, levofolinate calcium; CPT-11, irinotecan; MMC, mitomycin C; PTX, paclitaxel; IL-2, interleukin-2. *Dendritic cells pulsed with carcinoembryonic antigen-derived, HLA-A24-restrictid peptide (CEA652) were administered intradermally and subcutaneously.

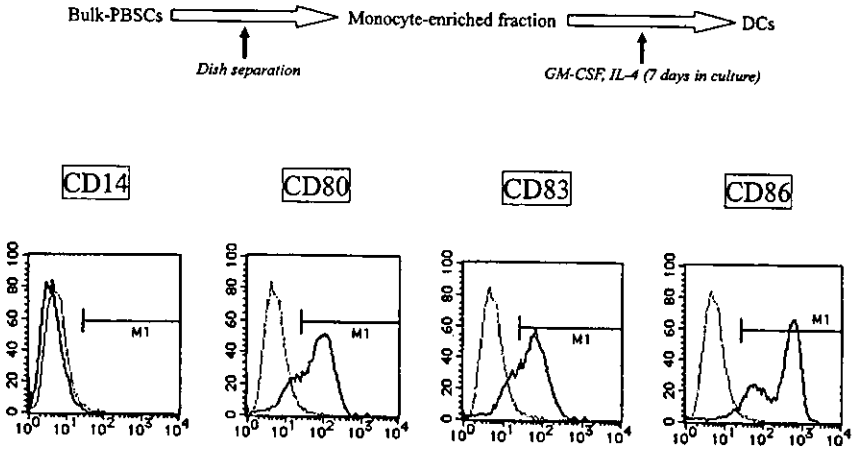

Figure 5. Surface markers of dendritic cells induced *in vitro* with GM-CSF and IL-4 from monocytes in cryopreserved bulk-PBSCs (Patient 5). Cryopreserved bulk-PBSCs were thawed rapidly, suspended in 50 ml of complete medium, and plated in cell culture flasks. The flasks were incubated in a 5% CO_2 incubator at 37°C for 2 h, and non-adherent cells were removed. Adherent cells (monocyte-enriched fraction) were cultured in complete medium containing 1,000 U/ml each of GM-CSF and IL-4. After culturing for 7 days, DCs were harvested, washed twice, and their surface markers were analyzed by flow cytometry (FACS Calibur and CellQuest softwar). Immunofluorescent staining was performed using the following monoclonal antibodies: FITC-conjugated anti-CD14, PE-conjugated anti-CD80, anti-CD83 and anti-CD86. PBSCT, peripheral blood stem cell transplantation; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-4, interleukin-4.

weeks of 100 mg/day of S-1 followed by 2 weeks rest) were given, but the serum CEA level increased to 810 ng/ml. There was no discernible evidence of recurrence on imaging examinations. HDC with PBSCT was then given as a second-line post-operative chemotherapy.

High-dose chemotherapy with PBSCT. HDC with PBSCT was administered in June, 2000. Cumulative doses of 600 mg/m^2 of VP16, 80 mg/m^2 of EPI, and 120 mg/m^2 of CDDP were given over the course of 3 days. PBSCT was done 4 days after completion of HDC. The nadir of the WBC was 500 /μl (day 14), and that of the neutrophil count was 0 /μl. Ten units of platelets were transfused on days 11 and 13. However, hematopoietic recovery after PBSCT was very prompt. The interval from PBSCT to recovery of the WBC to above 2,000 /μl was 8 days and the interval to recovery of the PLT to above 50,000 /μl

was 11 days (Fig. 3). After therapy, the serum CEA level fell to 161 ng/ml again, but did not fall below the upper limit of normal, suggesting that the cancer was refractory to treatment.

We gave the patient IV 5-fluorouracil (5-FU)-based and irinotecan (CPT-11)-based chemotherapy. However, multiple, bilateral lymph node metastases to the cervical and axillary regions became obvious and spread rapidly, with a steep rise in the serum CEA level. The level reached 2,550 ng/ml in April, 2001, 10 months after HDC with PBSCT. We decided to give the patient another type of cell therapy, using cryopreserved bulk-PBSCs, i.e. DC-based immunotherapy with CEA-derived CTL epitope.

DC-based immunotherapy with CEA-derived CTL epitope. The patient received 2 courses of the CEA-DC vaccine followed by systemic administration of IL-2. DCs were induced *in vitro* from monocytes in cryopreserved bulk-PBSCs remaining after transplantation. FACS analysis showed that the DCs lacked surface expression of CD14, but had high surface expression of costimulatory molecules (CD80, CD86) (Fig. 5). The therapy was carried out safely without any toxicity, except for mild fever caused by continuous administration of IL-2. The results of DTH skin tests, negative before vaccination, became positive after the vaccinations. The serum CEA level dropped to 1,500 ng/ml, but vaccine therapy could not be continued owing to depletion of the cryopreserved bulk-PBSCs.

Thereafter, paclitaxel (PTX), newly approved in Japan for gastric cancer at that time, was given weekly. However, at the end of September, 2001 the patient suddenly died of suffocation caused by growth of cervical lymph node metastases (Fig. 4).

Discussion

In the quest to find a new combination of anticancer drugs more effective than 5-FU-based regimens, Preusser *et al* developed EAP (1). Although nearly 20 years have elapsed since its initial report, EAP remains one of the most active combination regimens for advanced gastric cancer. The complete response (CR) rate with EAP is high (2,3), even as compared with more recent regimens including newly developed anticancer agents (21,22). The major drawback of EAP is WHO grade 4 myelotoxicity, occurring at an incidence of about 20% (2,3,23). Relatively high treatment-related mortality among elderly patients or those with poor PS has limited the use of EAP (4,5), even after the introduction of CSFs (GM-CSF, G-CSF) (10).

Hematopoietic stem cell transplantation is the most reliable means of protecting bone marrow from the adverse effects of intensive chemotherapy. Because bone marrow collection is not feasible in patients with advanced solid cancers, the use of PBSCT has rapidly increased. PBSCT offers the additional advantages of faster hematopoietic recovery and immune reconstitution than BMT (24,25). PBSCs are mobilized during hematopoietic recovery from the severe myelosuppression caused by intensive chemotherapy (26). Mobilization and harvesting of PBSCs after the first course of EAP may allow patients given PBSCT rescue therapy to safely receive HDC or a second course of EAP.

We attempted to harvest PBSCs after EAP in 5 patients with chemotherapy-naïve advanced gastric cancer. Two different assays were used to accurately enumerate hematopoietic stem cells included in harvested mononuclear cells (bulk-PBSCs), flow cytometry and hematopoietic colony formation assay. The results of colony formation assay (CFU-GM assay), provide indices of both the quality and quantity of the harvested stem cells and positively correlate with the number of $CD34^+$ cells on flow cytometric analysis (27). At least $2x10^5$/kg body weight CFU-GM or $2x10^6$/kg body weight $CD34^+$ cells are required to safely perform PBSCT after marrow-ablative chemotherapy (17,28). We described the course of bone marrow suppression after EAP in each of our 5 patients and showed that sufficient numbers of both $CD34^+$ cells and CFU-GM to permit safe PBSCT could be harvested by a single leukapheresis procedure in 4 patients, including a 72 year-old man (Patient 3). However, in the youngest patient, who had good PS (PS 0) (Patient 4), the neutrophil nadir was not as low as that in the other patients, and PBSCs could not be mobilized. Such patients may require a slightly more intensive, modified EAP regimen, incorporating higher doses of ADM to mobilize PBSCs, as recommended by Reichle *et al* (15).

HDC is an interesting concept for the management of malignant diseases sensitive to cytotoxic drugs and has shown considerable success against hematologic malignancies. However, the effectiveness of HDC for solid cancers, especially those arising in the gastrointestinal tract, remains a matter of debate (29). Several groups of investigators have used EAP-related HDC to treat advanced gastrointestinal cancers. Ajani *et al* gave high-dose EAP with GM-CSF support to 26 patients with adenocarcinoma of gastroesophageal junction or esophagus (10). Besides this report, 3 groups of investigators have examined the response to HDC with rescue therapy by hematopoietic stem cell transplantation (11,13-15) (Table IV). Although one study focused on high-dose EAP with BMT (11), the other two evaluated high-dose MCEB with PBSCT, using EAP as induction chemotherapy to mobilize PBSCs (13-15). The reasons for using high-dose MCEB instead of high-dose EAP for EAP-sensitive cancers were not specified in either of the latter reports. A combined total of 31 patients in the latter two studies received HDC with PBSCT as pre-operative chemotherapy, and no treatment-related death was reported. Moreover, 19 patients responding to pre-operative chemotherapy received surgery, and the patients with locally advanced disease and pathological evidence of a CR to HDC had prolonged disease-free survival (14,15).

In view of the conditions required for BMT, HDC with stem cell transplantation should be indicated in patients with earlier disease, associated with minimal tumor burden and the least likelihood of drug resistance (30). Before or immediately after surgery is considered the optimal time for HDC with stem cell transplantation. In our patient who received post-operative high-dose EAP with PBSCT (Patient 5), HDC with stem cell transplantation had to be delayed because of catheter-related infection and leakage of the esophagojejunostomy after surgery. These factors might have adversely affected survival. Hematopoietic recovery after PBSCT was very prompt, and HDC could be administered safely, with no severe complications in this patient. Two of the other 4 patients in this study had a PR to EAP, with mobilization of sufficient numbers of PBSCs. These patients could not receive HDC with PBSCT, either because of prolonged bone marrow suppression and sudden

Table IV. EAP-related high-dose chemotherapy studies of advanced gastric cancer.

Study (Reference)	No. of patients given high-dose chemotherapy	Induction chemotherapy	High-dose chemotherapy	Bone marrow support
Ajani *et al* (10)	26	-	High-dose EAP in 3 days ETP: 450 mg/m^2 ADM: 60 mg/m^2 CDDP: 105 mg/m^2	GM-CSF
Suzuki *et al* (11)	10	-	High-dose EAP in 3 days ETP: 1,200 mg/m^2 ADM: 80 mg/m^2 CDDP: 120 mg/m^2	BMT
Berdel *et al* (13,14)	5	Original EAP in 8 days ETP: 360 mg/m^2 ADM: 40 mg/m^2 CDDP: 80 mg/m^2	High-dose MCEB in 5 days MMC: 10 mg/m^2 CDDP: 160 mg/m^2 ETP: 1,000 mg/m^2 BCNU: 300 mg/m^2	PBSCT
Reichle et al (15)	26	Modified EAP in 3 days ETP: 360 mg/m^2 ADM: 80 mg/m^2 CDDP: 80 mg/m^2	High-dose MCEB in 4 days MMC: 10 mg/m^2 CDDP: 150 mg/m^2 ETP: 1,500 mg/m^2 BCNU: 300 mg/m^2	PBSCT

ETP, etoposide; ADM, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; BCNU, carmustine; GM-CSF, granulocyte/macrophage colony-stimulating factor; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation.

tumor regrowth after treatment (Patient 2) or because of grade 3 nausea and vomiting refractory to all kinds of anti-emetic drugs (Patient 1).

Our results and those of the 2 previous studies of high-dose MCEB with PBSCT (13-15) indicate that the original EAP regimen mobilizes PBSCs in most patients with chemotherapy-naïve advanced gastric cancer. Available findings also suggest that HDC (high-dose EAP or high-dose MCEB) with PBSCT is an effective and safe treatment for advanced gastric cancer, especially locally advanced disease without distant metastasis. Because there were 2 treatment-related deaths among the 26 patients given high-dose EAP with GM-CSF support by Ajani *et al* (10), increasing the dose intensity of EAP solely through the use of CSFs appears inadvisable.

Similar to studies by Suzuki *et al* (11), we attempted to mainly increase the dose intensity of ETP in EAP. However, even the dose of ETP used by Suzuki *et al*, two-fold higher than the dose in our study, was subablative (31). We agree with Suzuki *et al* that the pathophysiology of gastrointestinal cancer differs from that of other solid tumors. Gastrointestinal cancer is associated with much higher frequencies of infection and bleeding, as well as poorer nutritional status. Long-term aplasia carries considerable risk in patients with gastric cancer, especially after abdominal surgery (11). In addition, marrow-ablative HDC with PBSCT as the standard therapy for solid cancer has only been restricted to germ cell tumors in the definite, well-designed clinical trials (32). Therefore, it may be unreasonable to apply marrow-ablative EAP to gastric cancer, and PBSCT should be used as an adjuvant therapy for the safe tandem repeat of original or subablative modified EAP.

Besides PBSCT, rapid progress has recently been made in immune cell therapy. Tumor-specific active immunotherapy (cancer vaccine therapy) with DCs is a very promising new strategy, currently used to treat various types of cancer. DCs are professional antigen-presenting cells integral to the initiation of T-cell immunity. These cells can be induced *in vitro* from peripheral blood monocytes in the presence of GM-CSF and IL-4 (33). The advantage of active immunotherapy with DCs as compared with passive immunotherapy is the potential to induce long-lasting anamnestic T-cell responses (34).

We started a clinical trial of DC-based active immunotherapy with CEA652 peptide for advanced gastrointestinal tract cancer in 1998. In that trial, we showed that G-CSF-mobilized mononuclear cells include a large number of activated monocytes with significantly increased expression of major histocompatibility complex molecules. Potent DCs for clinical use can be induced efficiently from these activated monocytes (19,20). Other investigators have also confirmed the feasibility of obtaining both CD34$^+$ hematopoietic stem cells and DCs with potent functional activity from the same leukapheresis products derived from G-CSF-primed patients with cancer (35,36). As shown in Table III, most bulk-PBSCs harvested after intensive HDC followed by treatment with G-CSF consist of lymphocytes and monocytes activated to produce hematopoietic cytokines (37,38). It is therefore likely that immune cell therapies with these cells, given sequentially after PBSCT, would enhance the anti-tumor effect. In fact, some investigators have combined PBSCT with adoptive transfer of *in vitro* activated lymphocytes or DC-based active immuno-therapy in patients with hematological malignancies (39-41). These strategies have been shown to be effective against residual disease in some patients.

We used both PBSCT and DC-based active immunotherapy to treat a patient who had advanced gastric cancer with CEA

production. To our knowledge, this is the first report to describe the use of multimodal cell therapy with PBSCs in a patient with advanced gastric cancer. After immunotherapy, the serum CEA level declined in association with positive DTH skin test results to CEA652 peptide, suggesting that the tumor had responded to immunotherapy (19,20). However, the response was short-lived, with no definitive evidence of tumor shrinkage. Our pilot case study will hopefully encourage the further refinement and development of immune cell therapy in combination with PBSCT for advanced gastric cancer (42).

In conclusion, our study delineated the courses of bone marrow suppression after EAP in 5 patients with chemotherapy-naïve advanced gastric cancer. Sufficient numbers of PBSCs to permit safe transplantation were mobilized and harvested in 4/5 patients studied. Multimodal cell therapy with PBSCs is considered a feasible and promising treatment strategy for advanced gastric cancer. The development of novel intensive treatment strategies combining chemotherapy and immunotherapy will hopefully lead to the complete regression of cancer and prolonged long-term survival in young patients with advanced gastric cancer. Further investigation is warranted.

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Recognition of Epstein-Barr virus-associated gastric carcinoma cells by cytotoxic T lymphocytes induced *in vitro* with autologous lymphoblastoid cell line and LMP2-derived, HLA-A24-restricted 9-mer peptide

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Abstract. Epstein-Barr virus (EBV) is associated with several types of malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and gastric carcinoma. Previous reports have suggested that EBV-related antigen-targeting immunotherapy is one of the promising approaches for the treatment of these malignancies other than gastric carcinoma. EBV-associated gastric carcinoma (EBVaGC) has been shown to express Epstein-Barr virus nuclear antigen 1 (EBNA1) and latent membrane protein 2 (LMP2). In the present study, DNA and mRNA freshly isolated from tumors of patients with gastric cancer were subjected to polymerase chain reaction (PCR) using EBV-specific primers and reverse transcription (RT)-PCR specific for LMP2 transcripts. EBV-specific region was identified in genomic DNA isolated from cancerous tissues in 22% of gastric cancer patients. LMP2 mRNA was also detected in 3 out of these 5 DNA positive samples tested. To investigate the feasibility of specific immunotherapy for EBVaGC, we induced cytotoxic T lymphocytes (CTLs) from peripheral blood lymphocytes using two kinds of antigen-presenting cells (APCs) such as autologous lymphoblastoid cell line (LCL) and LMP2-derived peptide-pulsed dendritic cells (DCs). The cytotoxicity of these CTLs against peptide-pulsed targets was examined by standard ^{51}Cr release assay and interferon (IFN)-γ production assay. We further assessed the recognition of tumor cells endogenously expressing LMP2 by these T cells. T cells induced by peptide-loaded DCs and autologous LCL efficiently lysed peptide-pulsed targets. Furthermore, these T cells could recognize not only tumor cells transfected with LMP2, but also LMP2-positive gastric cancer cells which were successfully isolated and cultured from specimens obtained by surgery. Collectively, sensitization of peripheral blood lymphocytes with LMP2-derived peptide was able to induce CTL response against EBVaGC cells. Thus, EBVaGC is susceptible for the LMP2-targeting immunotherapy.

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Key words: Epstein-Barr virus-associated gastric carcinoma, immunotherapy, $LMP2_{419-427}$ peptide, lymphoblastoid cell line

Introduction

A wide variety of malignancies including Burkitt's lymphoma, Hodgkin's disease (1), undifferentiated nasopharyngeal carcinoma (2,3), post-transplant lymphoproliferative disease (PTLD) (4) and gastric carcinoma are associated with Epstein-Barr virus (EBV) (5,6). EBV-associated gastric carcinoma (EBVaGC) (7-12) has been demonstrated to account for 7-10% of all gastric carcinomas in Japan (13,14). Since there are >100,000 new patients with gastric carcinoma in Japan (15), the number of patients with EBVaGC is considerable.

Like other herpes viruses, EBV induces long-lasting cytotoxic T lymphocyte (CTL) memory (5). In fact, EBV-specific CTL precursors can be reactivated from the peripheral blood of EBV-seropositive individuals by several types of stimulation *in vitro* (5). Thus, to recall and boost T cell responses specific for EBV in EBVaGC patients could lead to the elimination of tumors.

EBV-transformed lymphoblastoid cell line (LCL) and cells from PTLD express all the latent proteins of EBV: six nuclear antigens [Epstein-Barr virus nuclear antigen (EBNA) 1, 2, 3A, 3B, 3C, and LP] and two membrane proteins [latent membrane protein (LMP) 1 and 2] (5). These latent proteins of EBV display hierarchical immunogenicity. Malignant cells in PTLD express the full set of EBV antigens, including the highly immunogenic EBNA3A, 3B, and 3C (16,17). Hence, PTLD is highly immunostimulatory. Rooney *et al* have clearly demonstrated that such EBV-associated disease can be effectively treated by transfer of EBV-specific CTLs that have been generated *in vitro* using donor LCL as stimulator

cells (17). However, the susceptibility of EBV antigens to immunotherapy for EBVaGC remains unclear. Although EBVaGC has been shown to express EBNA1 and LMP2 (5), EBNA1 may not be a suitable target due to its internal Gly/Ala repeat domain encompassed by EBNA1 protein which can prevent EBNA1 from processing by the classical HLA class I pathway (18,19). Thus, we focused on LMP2 as a target molecule for immunotherapy of gastric cancer in the current study.

Some immunodominant LMP2 epitopes restricted by several alleles of human leukocyte antigens (HLA) -class I have been identified. Because HLA-A24 is present in 60% of the Japanese population and in some Caucasians, we chose $LMP2_{419-427}$ peptide (TYGPVFMCL) restricted with HLA-A24 (20). To induce effective CTLs, we used a mixture of $LMP2_{419-427}$ peptide-loaded dendritic cells (DCs) and $LMP2_{419-427}$ peptide-loaded LCL as antigen-presenting cells (APCs) as well as LCL alone. Generated CTLs were tested for cytotoxicity against peptide-pulsed targets and LMP2-expressing tumor cells including primary cultured gastric cancer cells. To our knowledge, this is the first report to show that EBVaGC cells can be recognized by CTLs *in vitro* that are successfully induced with autologous LCL and LMP2-derived peptide.

Materials and methods

Patients and PBMCs from a healthy donor. Peripheral blood mononuclear cells (PBMCs) were isolated from a leukapheresis sample obtained from a healthy donor, FH01 (HLA-A24, B48, B52, DR14, DR15, EBV antibody titers: positive), by Ficoll-Paque (Pharmacia, Piscataway, NJ). Other subjects were 18 Japanese patients aged 55-86 years (13 men, 5 women) who had undergone resection of primary gastric carcinoma. Written informed consent was obtained from the healthy donor and from all patients before surgery.

Cell lines. LCL was generated *in vitro* by transforming B cells from FH01 with the standard EBV isolate B95.8 (21,22), kindly supplied by Dr Kenzo Takada (Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan). K562 (chronic myelogenous leukemia) (23) was obtained from the Japanese Collection of Research Bio-sources (JCRB). 888mel (melanoma cell line, HLA-A24+) (24) was kindly provided by Dr Yutaka Kawakami (Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan). All cell lines were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal calf serum (Nichirei, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO).

Primary culture of gastric carcinoma tissues. The remaining sections of carcinoma tissue after general pathological examinations were cut into small pieces (<1 mm^3), put into plastic dishes, and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 10 days, a half of the medium was changed; subsequently the medium was changed once per week. After confluent cultures had developed,

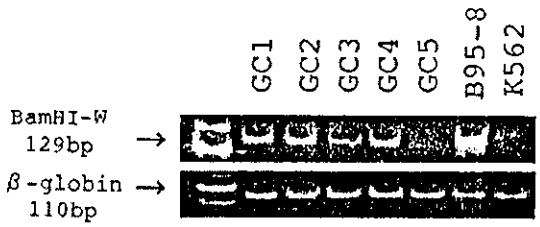

Figure 1. Detection of EBV DNA in gastric carcinoma tissues by PCR analysis. Total cellular DNA extracted from 5 cases (GC1-GC5) of gastric carcinoma and from B95-8 (EBV-positive) and K562 (EBV-negative) cells were subjected to PCR. Arrows indicate the specific PCR products with their predicted sizes. One of the EBV-negative gastric carcinomas, GC5, is shown in the same blot.

the cell cultures were tested for LMP2 expression by immunostaining with rat monoclonal antibodies specific for LMP2 (25) using Histofine Simple Stain MAX-PO kits according to the manufacturer's instructions (Nichirei, Tokyo, Japan). LMP2 antibodies and Plasmid LMP2/pSG5 were kindly supplied by Dr Richard Longnecker (Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, IL).

BamHI-W PCR. DNA samples were extracted from the resected cancer tissues and tested for EBV by *Bam*HI-W polymerase chain reaction (PCR). EBV-specific primers (5'-CCAGACA GCAGCCAATTGTC-3' and 5'-GGTAGAAGACCCCCTCT TAC-3') were used to amplify the 129 bp fragment in the *Bam*HI-W region (26). To test whether total cellular DNA had been extracted, the nucleotide sequence of the human ß-globin gene was amplified with the use of an aliquot of total DNA. B95-8 cells were used as a positive control, and K562 cells were used as a negative control. PCR products were run on 5% polyacrylamide gels. PCR was performed with a KOD Dash PCR kit (Toyobo, Osaka, Japan).

RT-PCR and detection of amplified products. Total RNA was extracted using Isogen reagent according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan). Reverse transcription (RT)-PCR was performed with a BcaBest RNA PCR kit (Takara Bio, Shiga, Japan) (25). LMP2A-specific primers (5'-ATGACTCATCTCAACACATA-3' and 5'-CATGTTAG GCAAATTGCAAA-3') were used for amplification of the 280 bp fragment (26,27). cDNA from tumor tissues and EBV-negative K562 cells were subjected to 35-40 cycles of amplification. cDNA from EBV-positive B95-8 was used as a positive control and subjected to 20-25 cycles of amplification. To confirm that total RNA had been extracted, human ß-tublin mRNA was amplified using an aliquot of cDNA. Specific primers (5'-TGGATCTAGAACCTGGGACCAT-3' and 5'-ACCATGTTGACTGCCAACTTGC-3') were used for amplification of 577 bp in the ß-tublin mRNA, and amplified products were visualized by ethidium bromide staining.

Generation of LCL-induced CTLs. FH01-PBMCs ($2x10^6$) were co-cultured with irradiated (100 Gy) autologous LCL (FH01-LCL) in 24-well tissue culture plates (responder:APC = 40:1). On day 10, the cultures were replated at $2x10^5$ cells per