responses. In a previous study, we also made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.[7,10] In addition, we have recently developed a new immunotherapeutic approach for HCC using DC infusion performed during TAE, showing the potential to enhance tumor-specific immune responses.[7]

In the current study, we first attempted to identify the effect of TAE for tumor-specific T-cell responses in patients with HCC. Next, we examined the additional effects of DC infusion to the tumor site after TAE. Finally, we analyzed the relationship between clinical characteristics of patients and T-cell responses after TAE and evaluated whether the activation of tumor-specific T-cell responses can prevent HCC recurrence.

Material and Methods

Patient population

The study examined 33 patients with HCC, consisting of 25 men and 8 women ranging from 48 to 83 years old with a mean age of 66 ± 9 years. Twenty patients were treated by TAE. Thirteen patients were treated by TAE with DC infusion as a part of clinical study, which was approved by ethical committee of Kanazawa University Graduate School of Medical Science and registered in September 2003. The patients who received TAE with DC infusion were selected according to the criteria we previously reported.[7] All subjects were negative for Abs to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration.

Treatment of hepatocellular carcinoma

HCCs were detected by imaging modalities such as dynamic CT scan, MR imaging and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens, surgical resection or autopsy in 18 cases. For the remaining 15 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.[33] The tumor size was categorized as "small" ($\leq$2 cm) or "large" (>2 cm), and tumor multiplicity was categorized as "multiple" ($\geq$2 nodules) or "solitary" (single nodule). The TNM stage was classified according to the Union Internationale Contre Le Cancer (UICC) classification system (6th version).[34]

Twenty patients were treated by TAE as previously described.[19,35] In brief, after evaluation of the feeding arteries and surrounding vascular anatomy, a microcatheter (Microferret, Cook, Bloomington, IN) was inserted into the segmental or subsegmental artery with a coaxial method using a 0.016-inch guidewire (Radifocus GT wire, Terumo, Tokyo, Japan). A mixture of the anticancer drug and iodized oil was administered, and the feeding artery was embolized with gelatin sponge particles (Gelfoam; Pharmacia Upjohn, Kalamazoo, MI). The mixture of anticancer drug and iodized oil contained 10–30 mg of Epirubicin (Farmorubicin; Kyowa Hakko Kogyo, Tokyo, Japan), 1–3 ml of iodized oil (Lipiodol Ultra Fluide) and 0.5–1.0 ml of iohexol (Omnipaque 300).

Preparation and injection of autologous DCs

DCs were generated as previously described.[7] In 6 patients, DCs were pulsed with 0.1 KE/ml OK-432 (Chugai Pharmaceutical, Tokyo, Japan), which is a biological response modifier derived from the weakly virulent Su strain of Streptococcus pyogenes,[36,37] for 3 days before injection. The cells were harvested for injection; 5×10^6 cells were reconstituted in 5-ml normal saline containing 1% autologous plasma, mixed with gelatin sponge particles and infused through an arterial catheter following iodized oil injection during TAE.

After TAE or TAE with DC infusion, 26 patients received percutaneous tumor ablation by ethanol injection (PEIT), microwave coagulation (MCT) or radiofrequency (RF). Twenty-one patients were diagnosed with complete necrosis of the tumor lesion using dynamic CT after the completion of treatment. Follow-ups were conducted at outpatient clinics using blood tests and dynamic CT every 3 months for 1 year.

Laboratory and virologic testing

Blood samples were tested for HBsAg and HCVAb by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of PBMC from patients was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda. The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathologic study of primary liver cancer.[38] The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.*[39]

Interferon-γ enzyme-linked immunospot assay

The prevalence of tumor antigen-specific T cells was determined by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis (Mabtech, Nacka, Sweden) as previously described.[10,40] HLA-A24-restricted AFP-derived peptides (10 μg/ml), which were AFP_{357} (EYSRRHPQL), AFP_{403} (KYIQESQAL) and AFP_{434} (AYTKKAPQL),[10] and 20 μg/ml AFP derived from human placenta (Morinaga Institute of Biological Science, Yokohama, Japan, purity >98%) were added directly to the wells. These 3 AFP-derived peptides could induce CTLs showing cytotoxicity against hepatoma cells and were frequently recognized by PBMCs of patients with HCC as we previously reported,[10] and therefore, we selected them as an immunogenic peptide. The HLA-A24-restricted AFP and CMV-derived peptides were used only for HLA-A24 or A23 positive patients. Other tumor antigen-derived peptides consisted of $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), $MRP3_{765}$ (VYSDADIFL), $hTERT_{167}$ (AYQVCGPPL), $hTERT_{324}$

(VYAETKHFL) and hTERT$_{461}$ (VYGFVRACL), which we previously reported that they were useful for analyzing host immune responses to HCC.[40,41]

PBMCs were added to the wells at 3×10^5 cells/well. In the assay using PBMC depleted $CD4^+$ or $CD8^+$ cells, the number of cells was adjusted to 3×10^5 cells/well after the depletion. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system using CD4 or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. After the depletion, 1×10^6 cells were stained with CD4 and CD8 antibodies (Becton Dickinson, Tokyo, Japan) and analyzed by FACSCalibur (Becton Dickinson, Tokyo, Japan) to confirm the ratio of $CD4^+$ and $CD8^+$ cells. Data analysis was undertaken with CELLQuest^{TM} software (Becton Dickinson, San Jose, CA).

Plates were analyzed with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least 2-fold greater than the number of spots in the absence of antigen. Negative controls consisted of incubation of PBMCs with a peptide representing an HLA-A24-restricted epitope derived from HIV envelope protein (HIVenv$_{584}$) and were always <5 spots per 3×10^5 cells.[42] The positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide (CMVpp65$_{328}$).[43] All peptides used in this study were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). ELISPOT analysis was performed before and 2–4 weeks after TAE. In patients receiving additional treatment for complete ablation of tumor, analysis was performed just before the additional treatment. An increase of antigen-specific T cells was defined as significant when T-cell responses changed to positive or if the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment.

Statistical analysis

Unpaired Student's *t*-test was used to analyze the effect of variables on immune responses in patients with HCC. Fisher's exact test (2-sided *p*-value) was used to analyze the frequency of positive immune responses in patients between with TAE and TAE with DC infusion.

Results

T-cell responses to AFP in the patients who received TAE

The frequency of AFP-specific T cells before and after TAE was tested *ex vivo* in an IFN-γ ELISPOT assay. The serum AFP level and number of peripheral lymphocytes and antigen-specific T cells are shown in Table 1. Before treatment, 2 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein in 20 patients (Patients 1–20). After treatment, a T-cell response to AFP-derived peptides and protein was detected in 4 and 3 patients, respectively.

When an increase of antigen-specific T cells was defined as significant if T-cell responses changed to positive or the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment, 6 of 20 (30%) patients (Patients 4, 6, 7, 11, 18 and 20) showed a significant increasing of AFP-specific T-cell frequency after treatment. It was observed even in the patient (Patients 6, 7 and 18) who had no T cells specific to corresponding AFP-derived peptides before treatment. When a decrease of antigen-specific T cells was defined as significant if T-cell responses changed from positive to negative or the number of spots detected after TAE was less than half of the number of spots detected before treatment, 4 of 20 (20%) patients (Patients 5, 14, 15 and 16) showed a significant decreasing of AFP-specific T-cell frequency after treatment.

AFP-specific IFN-γ-producing T cells were also analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system, and the results were confirmed by flow cytometric analysis (Fig. 1*a*). After depletion of $CD4^+$ or $CD8^+$ cells, the ratio of each cell population was decreased to less than 0.1% of PBMCs. The IFN-γ ELISPOT assay showed that IFN-γ-producing T cells against AFP consisted of both $CD8^+$ and $CD4^+$ cells (Fig. 1*b*).

To confirm the effect of TAE for host immune responses to HCC, we also examined the frequency of tumor antigen-specific T cells in 4 patients (Patients 5, 8, 10 and 14) using MRP3- or hTERT-derived peptides that we previously identified as useful for analyzing host immune responses to HCC.[40,41] A significant increasing of MRP3- or hTERT-specific T-cell frequency was observed in all patients after TAE (Table 2).

T-cell responses to AFP in the patients who received TAE with DC infusion

In 13 patients receiving TAE with DC infusion (Patients 21–33), 2 patients showed a specific T-cell response with AFP-derived peptides and 2 patients with protein before treatment (Table 3). After treatment, 8 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein.

Next, we compared TAE with DC infusion with TAE alone regarding the effect to AFP-specific immune response. Table 4 shows the clinical features of patients with HCC who received TAE and TAE with DC infusion and they were not statistically different except liver function.

The frequency of patients who showed both positive and increasing T-cell response with AFP-derived peptides or protein after treatment was significantly higher in patients receiving TAE with DC infusion than in those receiving TAE alone ($p = 0.04$) (Fig. 2*a*). On the other hand, the frequency of patients who showed both positive and increasing T-cell

Table 1. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE

				Before treatment								After treatment							
Patient	HLA	Additional treatment	Complete ablation	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT
1	A2	RF	C	<10	1,600	ND	ND	ND	1	ND	0	<10	1,400	ND	ND	ND	0	ND	1
2	A26,A31	RF	C	61	1,700	ND	ND	ND	0	ND	**13**	23	900	ND	ND	ND	0	ND	0
3	A11,A26	No	–	100	1,700	ND	ND	ND	5	ND	1	50	1,500	ND	ND	ND	0	ND	0
4	A24	RF	C	18	700	0	7	0	6	0	**25**	16	500	1	**10**	1	1	2	**16**
5	A24,A33	RF	C	2,357	1,200	**13**	2	6	0	**13**	0	700	1,100	2	1	1	0	9	0
6	A24	RF	C	14	1,800	0	0	0	0	0	**42**	<10	1,400	**53**	**27**	**38**	**14**	**36**	**108**
7	A23,A33	No	–	96	500	0	0	0	5	**291**	0	138	800	**46**	0	0	3	**484**	0
8	A24,A26	No	–	142	600	1	0	0	0	0	0	126	500	2	0	0	0	**166**	1
9	A2,A24	RF	C	<10	700	6	1	0	0	9	0	<10	700	0	0	0	0	**32**	**15**
10	A24	PEIT	C	<10	1,300	8	4	8	8	**146**	5	<10	1,300	0	1	1	0	1	1
11	A24,A26	PEIT	N	18	1,100	0	0	0	1	ND	0	13	400	0	0	0	**15**	**10**	**55**
12	A24,A33	RF	N	11	800	3	2	0	4	**94**	**10**	11	700	0	0	0	0	**24**	0
13	A11,A24	PEIT	C	52	1,300	0	2	5	1	2	0	24	1,200	0	0	0	0	0	3
14	A24	RF	C	54	2,400	**25**	5	4	8	**12**	0	67	1,700	0	0	0	0	0	0
15	A2,A24	RF	N	62	1,200	0	3	0	**25**	2	3	14	800	0	0	0	8	0	0
16	A3,A24	RF	C	2,876	900	0	1	0	**13**	0	5	3,285	700	0	0	0	0	0	0
17	A24,A33	No	–	205	400	4	2	3	2	**26**	9	220	100	2	1	0	1	**39**	1
18	A24,A30	RF	C	18	1,100	4	0	3	8	**14**	7	13	900	1	**16**	1	5	**12**	0
19	A2,A24	RF	C	330	1,500	2	0	0	0	**18**	1	36	1,100	0	4	0	3	8	1
20	A2,A33	RF	C	10	1,400	ND	ND	ND	**10**	ND	**68**	<10	800	ND	ND	ND	**31**	ND	**101**

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; No, no treatment; C, completed; N, not completed; –, not determined; ND, not done. The bold letters show the positive responses in ELISPOT assays.

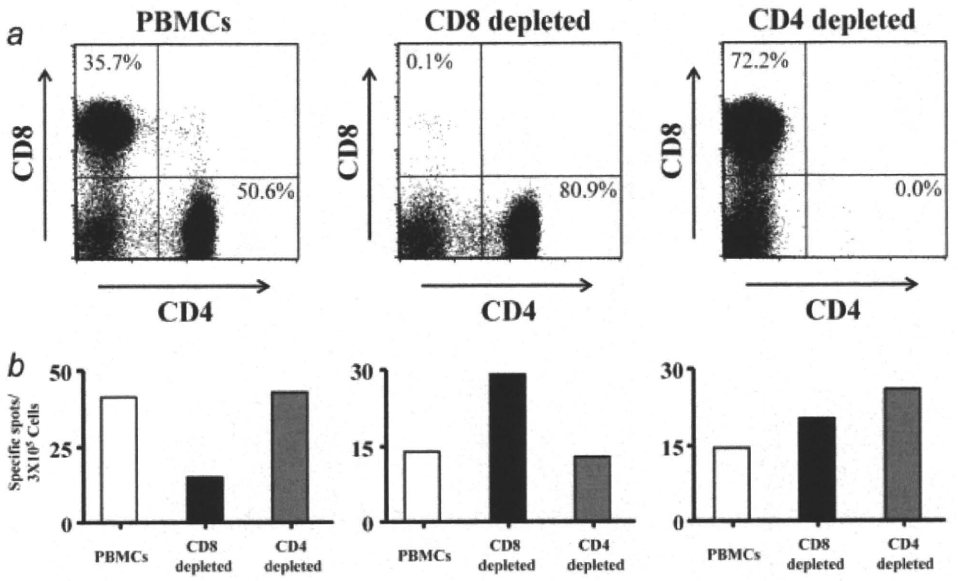

Figure 1. IFN-γ production of CD4- or CD8-depleted T cells against whole AFP. AFP-specific IFN-γ-producing T cells were analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system and the results were confirmed by flow cytometric analysis (*a*). IFN-γ ELISPOT assay using nontreated PBMCs and PBMC depleted $CD4^+$ or $CD8^+$ cells showed that T cells producing IFN-γ against whole AFP consisted of both $CD8^+$ and $CD4^+$ cells (*b*). Assays were performed in 5 patients and the representative result is shown.

Table 2. T cell response to other tumor antigen-derived peptides by ELISPOT assay before and after TAE

	Before treatment						After treatment					
Patient	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$
5	2	7	8	0	3.5	7.5	0	0	0	7	3	**35**
8	6	6	1	3	ND	ND	**17**	**18**	**22**	**18**	**14**	9
10	0	1	3	0	5	7	0	4	7	6	**11**	4
14	6	5	0	9	5	**13**	6	14	22	8	**10**	7

Abbreviation: ND, not done. The bold letters show the positive responses in ELISPOT assays.

response with CMV-derived peptide or tetanus toxoid was not different between the 2 groups (Figs. 2*b* and 2*c*).

In the comparison of the mean values of spots generated with AFP-derived peptides, protein, CMV-derived peptides or tetanus toxoid, no significant difference was observed between patients with TAE alone before and after treatment (Figs. 3*a*–3*d*). In contrast, the mean values of spots generated with AFP-derived peptides were significantly higher in patients after TAE with DC infusion than in those before treatment (Fig. 3*e*). The mean values of spots generated with protein, CMV-derived peptides or tetanus toxoid were not significantly different between patients before and after TAE with DC infusion (Figs. 3*f*–3*h*). Based on the above results, we considered that the main difference between TAE alone and TAE with DC infusion was the response to HLA-A24-restricted AFP-derived epitopes. Therefore, to analyze the difference between TAE alone and TAE with DC infusion more precisely, we selected the patients with HLA-A24 or A23 and compared the clinical parameters of both groups. However, there were no statistical differences except liver function in the 2 groups (Table 5).

Enhancement of AFP-specific T-cell responses and treatment outcome

To evaluate the effect of immune enhancement by TAE or TAE with DC infusion for the treatment outcome, we analyzed the clinical course of 17 patients who received complete ablation by additional RFA, PEIT or MCT after these treatments and could be followed up using dynamic CT every 3 months (Table 6). Seven patients showed increasing specific spots for AFP or AFP-derived peptides in ELISPOT assay after TAE. HCC recurrence within 3 months after complete ablation was observed in 3 patients who showed increasing AFP-specific T-cell responses after TAE. Furthermore, recurrence within 6 months after complete ablation was observed

Table 3. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE with DC infusion

				Before treatment								After treatment							
Patient	HLA	Additional treatment	Complete ablation	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT
21	A24	No	–	332	1,100	7	1	4	ND	**10**	ND	819	800	**11**	0	**10**	ND	**188**	ND
22	A24,A26	RF	N	341	700	0	**26**	5	ND	**68**	ND	237	500	ND	**59**	ND	ND	**81**	ND
23	A11,A24	No	–	41	600	0	2	5	1	2	0	43	400	0	0	0	0	0	3
24	A2,A24	MCT	C	1,260	800	3	8	7	ND	**19**	ND	614	1,300	**26**	4	7	ND	**12**	ND
25	A24,A33	RF	C	11	1,500	0	1	0	**31**	5	**15**	19	900	1	4	**15**	**26**	3	4
26	A24,A33	RF	C	<10	2,000	0	0	0	0	0	0	<10	1,700	0	**16**	0	0	0	0
27	A24,A26	RF	C	16	700	0	0	0	1	1	0	16	700	2	1	**15**	9	0	1
28	A11,A31	RF	N	31	800	ND	ND	ND	3	ND	0	33	700	ND	ND	ND	0	ND	0
29	A11,A33	No	–	<10	1,100	ND	ND	ND	0	ND	0	<10	700	ND	ND	ND	0	ND	1
30	A2,A11	RF	C	13	1,300	ND	ND	ND	8	ND	1	14	1,500	ND	ND	ND	**12**	ND	7
31	A24,A33	RF	C	1,014	800	0	0	0	0	1	0	15	300	0	0	**20**	0	0	0
32	A11,A24	RF	C	<10	1,000	3	3	**11**	**48**	**97**	0	10	1,200	**23**	**20**	**20**	**45**	**91**	**23**
33	A2,A26	RF	C	29	1,300	ND	ND	ND	0	ND	0	27	1,300	ND	ND	ND	0	ND	0

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; MCT, microwave coagulation therapy; C, completed; N, not completed; –, not determined; ND, not done. The bold letters show the positive responses in ELISPOT assays.

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Table 4. Patient characteristics

	Patients treated by TAE (n = 20)	Patients treated by TAE with DC (n = 13)	p-value[1]
Age (years)[2]	66.6 ± 7.8	65.7 ± 10.0	NS
Sex (M/F)	14/6	11/2	NS
HLA (A23 or 24/others)	16/4	9/4	NS
ALT (IU/l)	51.0 ± 47.4	86.9 ± 62.8	NS
Total bilirubin (g/dl)	1.3 ± 0.9	1.5 ± 0.9	NS
Albumin (g/dl)	3.7 ± 0.7	3.2 ± 0.6	NS
AFP level (ng/ml)	322.7 ± 793.0	239.8 ± 418.2	NS
Diff. degrees of HCC (well/moderate or poor/ND[1])	2/6/12	4/4/5	NS
Tumor size (small/large[3])	4/16	1/12	NS
Tumor multiplicity (multiple/solitary)	18/2	12/1	NS
TNM stage (I, II/III, IV)	19/1	11/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	15/5	10/3	NS
Liver function (Child A/B or C)	14/6	3/10	0.02
Etiology (HCV/HBV/others)	12/2/6	13/0/0	NS

[1]Abbreviations: NS, no statistical significance; ND, not determined. [2]Data are expressed as the mean ± SD. [3]Small: ≤2 cm, large: >2 cm.

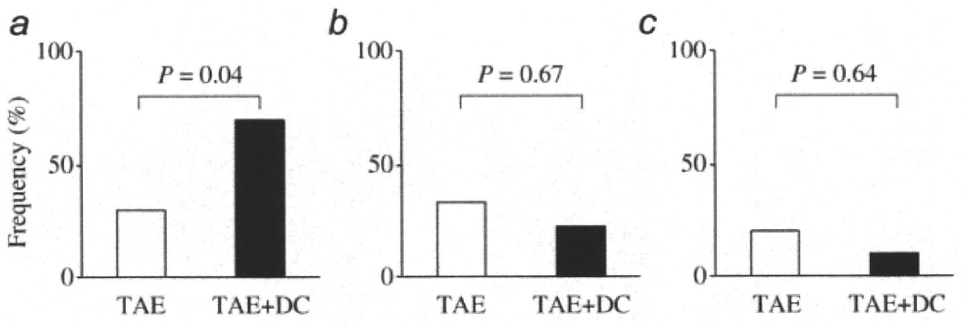

Figure 2. Frequency of the patients who showed enhancement of T-cell responses after treatment. The prevalence of antigen-specific T cells was determined by IFN-γ ELISPOT analysis using alpha-fetoprotein (AFP) and AFP-derived peptides (*a*), CMV pp65-derived peptide (*b*) or tetanus toxoid protein (*c*) in 20 and 13 patients with HCC who received TAE and TAE with DC infusion, respectively.

in 4 and 6 patients who did and did not show increasing AFP-specific T-cell responses, respectively.

Kinetics of AFP-specific T-cell responses before and after TAE

Next, we examined the kinetics of AFP-specific T cells in 8 patients who showed increasing frequency of IFN-γ-producing T cells against AFP or AFP-derived peptides after TAE. The frequency was examined by ELISPOT assay before and 2–4 weeks and 3 months after TAE. Thirteen kinds of AFP-specific T cells showed increasing frequency 2–4 weeks after TAE (Fig. 4); however, the increase was transient and most cell types decreased 3 months after TAE. Three patients showed more than 10 specific spots for AFP or AFP-derived peptides 3 months after TAE (Patients 6, 11 and 30). In analysis of the correlation between the maintenance of AFP-specific T-cell responses and HCC recurrence, 1 patient (Patient 6) had HCC recurrence after 6 months and 1 patient (Patient 30) did not show recurrence. Another patient (Patient 11) did not receive curative ablation and was not analyzed. There was no difference in the kinetics of AFP-specific T cells between patients who received TAE with and without DC infusion.

Discussion

In a previous study, we made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.[7,10] Similarly, as in our previous or other group's results,[8] we observed enhancement of AFP-specific immune responses in 6 of 20 patients with TAE alone in this study. The enhancement of tumor antigen-specific immune responses was also observed in the cases using MRP3- or hTERT-derived peptides.

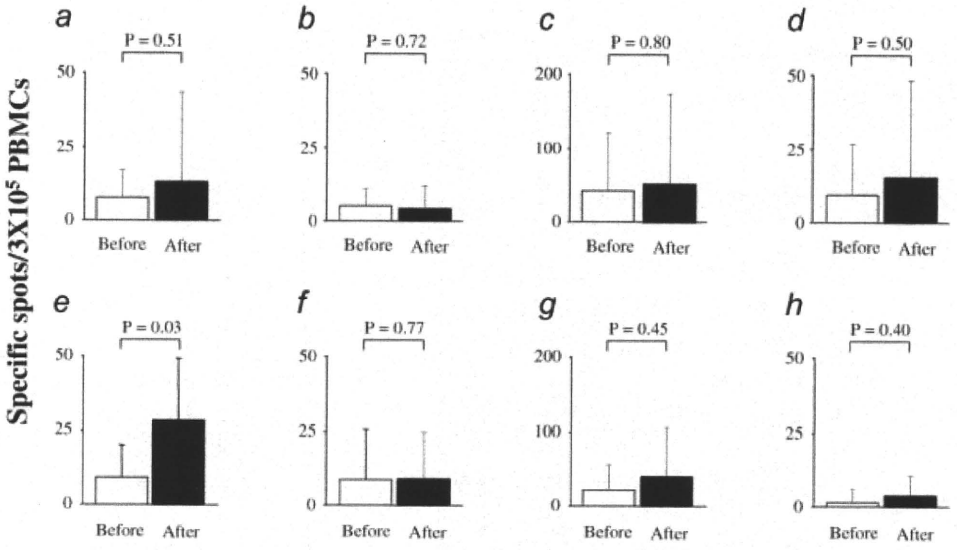

Figure 3. Comparison of direct *ex vivo* analysis (IFN-γ ELISPOT assay) before and after treatment of HCC. The assay was performed using PBMCs of patients who received TAE for AFP-derived peptides (*a*), AFP (*b*), CMV pp65-derived peptide (*c*) or tetanus toxoid protein (*d*). The same assay was performed using PBMCs of patients who received TAE with DC infusion for AFP-derived peptides (*e*), AFP (*f*), CMV pp65-derived peptide (*g*) or tetanus toxoid protein (*h*). AFP and CMV pp65-derived peptides were tested in only HLA-A24 or A23 positive patients. Data are expressed as the mean + SD of specific spots.

Table 5. Characteristics of the patients with HLA-A24 or A23

	Patients treated by TAE (n = 16)	Patients treated by TAE with DC (n = 9)	p-value[1]
Age (years)[2]	65.7 ± 7.8	67.8 ± 10.8	NS
Sex (M/F)	10/6	7/2	NS
ALT (IU/l)	55.9 ± 51.9	75.4 ± 53.0	NS
Total bilirubin (g/dl)	1.4 ± 0.8	1.4 ± 1.1	NS
Albumin (g/dl)	3.6 ± 0.7	3.1 ± 0.6	NS
AFP level (ng/ml)	392.1 ± 877.8	337.2 ± 477.1	NS
Diff. degree of HCC (well/moderate or poor/ND[1])	2/5/9	3/3/3	NS
Tumor size (small/large[3])	3/13	0/9	NS
Tumor multiplicity (multiple/solitary)	15/1	8/1	NS
TNM stage (I, II/III, IV)	15/1	7/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	13/3	8/1	NS
Liver function (Child A/B or C)	10/6	0/9	0.003
Etiology (HCV/HBV/others)	11/1/4	9/0/0	NS

[1]Abbreviations: NS, no statistical significance; ND, not determined. [2]Data are expressed as the mean ± SD. [3]Small: ≤2 cm, large: >2 cm.

The precise mechanism of this phenomenon is still unknown; however, in recent studies, several treatments to destroy tumor cells by necrosis and/or apoptosis have induced antitumor immune responses in animal models[14,44] and even in humans.[6–10] In the study of *in situ* tumor ablation, it is reported that tumor ablation creates a tumor antigen source for the induction of antitumor immunity.[9,44] In another study regarding photodynamic therapy (PDT),[45] it is reported that acute inflammation, expression of heat-shock proteins and providing tumor antigens to DCs caused by PDT induce tumor-specific immune responses.

Based on these results, we hypothesize that DC infusion with TAE can induce antitumor immune responses more effectively than TAE alone. According to DC research in recent years, successful enhancement of the antitumor immune response has been reported by intratumoral

Table 6. Enhancement of AFP-specific T cell response and treatment outcome

	Enhancement of AFP-specific T cell response	Recurrence, 3 months	Recurrence, 6 months
Patient 1	−	N	U
Patient 2	−	N	M
Patient 4	+	M	ND
Patient 5	−	N	M
Patient 6	+	N	U
Patient 9	−	N	M
Patient 10	−	N	N
Patient 13	−	N	N
Patient 14	−	N	N
Patient 16	−	N	M
Patient 19	−	N	U
Patient 24	+	U	ND
Patient 25	+	M	ND
Patient 26	+	N	N
Patient 30	+	N	N
Patient 31	+	N	N
Patient 33	−	N	N

Abbreviations: N, no recurrence; U, uninodular recurrence; M, multinodular recurrence; ND, not determined.

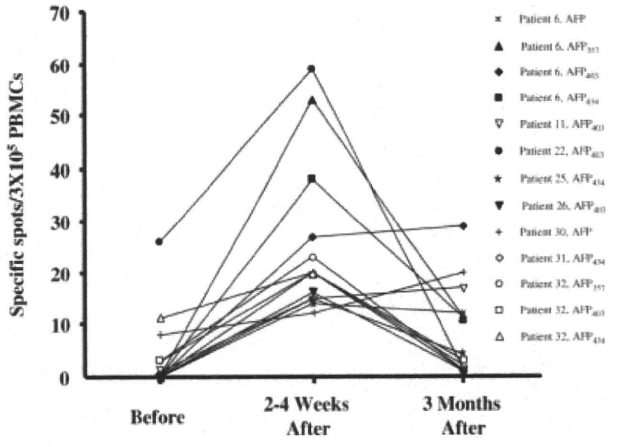

Figure 4. Kinetics of AFP-specific T-cell responses determined by IFN-γ ELISPOT assay before and after TAE. PBMCs were obtained before and 2–4 weeks and 3 months after TAE. Each graph indicates the kinetics of T cells specific for each antigen in each patient. Some patients received additional treatments as indicated in Tables 1 and 3 for a curative treatment after the measurement of T-cell responses at 2–4 weeks after TAE.

administration of DC in combination with tumor ablation.[46,47] Furthermore, immunotherapies using DC have been performed in patients with HCC and their antitumor effects are reported.[48–50] These results support our hypothesis and therefore, in the next step, we examined the immunological effects of DC infusion with TAE.

The comparison of frequency in patients who showed enhancement of AFP-specific immune responses revealed more frequency in patients with DC infusion than in those with TAE alone. On the other hand, there were no differences in the 2 groups in the comparison of frequency for patients who showed enhancement of CMV or TT-specific immune responses. These results suggest that DC infusion with TAE affects tumor-specific immune responses and that the effects are limited to the tumor area.

Some patients with TAE alone showed disappearance of AFP- or control antigen-specific T cells. Although the mechanism of this phenomenon is unknown, anticancer drugs used in TAE might suppress the immune responses, because most of the patients showed decreasing the number of lymphocytes after TAE. These results suggest that TAE alone might give a chance to enhance tumor-specific T-cell responses in only some patients. Further analysis using many more patients with TAE is necessary to make clear the differences in the patients with and without enhancement of T-cell responses. In contrast, disappearance of AFP- or control antigen-specific T cells was not observed in the patients with DC infusion, suggesting strong immunostimulating effect of this treatment.

In analysis of the association between the enhancement of AFP-specific T cells and clinical responses, no correlation could be shown, suggesting that enhancement of T-cell response associated with TAE or TAE with DC infusion may not have protective effect against HCC recurrence. To clarify the mechanism in more detail, we examined the kinetics of AFP-specific T-cell response. Increased frequency of AFP-specific T cells was transient and fell in 4 of 8 patients 3 months after treatment (Fig. 4). Similar to our results, Ayaru *et al.* also reported that the frequency of AFP-specific $CD4^+$ T cells fell in all patients by 1–3 months after TAE.[8] In addition, our results suggest that DC infusion with TAE is not effective to maintain the increased frequency of AFP-specific T cells.

Recent genome profiling studies of HCC show that HCC is a very heterogenous tumor.[51] Furthermore, HCC has multicentric carcinogenesis and develops at different time points. These characters of HCC may also be another reason for no correlation between the enhancement of AFP-specific T cells and clinical responses. The identification of many more tumor antigens and their T-cell epitopes is necessary for more precise analysis of the relationship between antitumor immune response and clinical response, and for immunotherapy.

In the recent study, it is reported that $CD8^+$ T-cell response to AFP is multispecific and AFP-specific IFN-γ-producing $CD8^+$ T cells are directed against different epitopes spreading over the entire AFP sequence with no single

immuno-dominant CD8$^+$ T-cell epitope.[52] Therefore, there is a limitation to our study, because the number of immunogenic AFP-derived peptides applicable in this study is small. However, the results of the present study suggest that TAE with DC infusion enhances the tumor-specific immune responses. Although these modified immune responses may not be sufficient to prevent HCC recurrence because the enhanced immune responses are transient and attenuate within 3 months, these results may contribute to the development of novel immunotherapeutic approach for HCC.

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BASIC RESEARCH

Cryoimmunologic Antitumor Effects Enhanced by Dendritic Cells in Osteosarcoma

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Abstract

Background We previously reported a limb-salvage technique by treating tumor-bearing bone with liquid nitrogen. We also reported systemic antitumor immunity was enhanced by cryotreatment in a murine osteosarcoma (LM8) model. We therefore combined the cryotreatment of tumor with dendritic cells to promote tumor-specific immune responses.

Questions/purposes We determined whether our technique could enhance systemic immune response and inhibit metastatic tumor growth in a murine osteosarcoma model.

Materials and Methods To evaluate activation of the immune response, we prepared six groups of C3H mice (80 mice total): (1) excision only, (2) dendritic cells without reimplantation of the cryotreated primary tumor, (3) reimplantation of the cryotreated primary tumor alone, (4) dendritic cells combined with reimplantation of the cryotreated primary tumor, (5) dendritic cells exposed to cryotreated tumor lysates without reimplantation of the cryotreated primary tumor, and (6) dendritic cells exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor. We then compared and verified the activation state of each group's antitumor immunity.

Results Mice that received dendritic cells exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor group had high serum interferon γ, reduced pulmonary metastases, and increased numbers of CD8(+) T lymphocytes in the metastatic areas.

Conclusions Combining tumor cryotreatment with dendritic cells enhanced systemic immune responses and inhibited metastatic tumor growth.

Clinical Relevance We suggest immunotherapy could be developed further to improve the treatment of osteosarcoma.

Each author certifies that he or she has no commercial associations that might pose a conflict of interest in connection with the submitted article.
Each author certifies that his or her institution has approved the animal protocol for this investigation, and that all investigations were conducted in conformity with ethical principles of research.
This work was performed at the Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University, and the Department of Orthopaedic Surgery, Faculty of Medicine, Oita University.

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Introduction

The standard treatment of osteosarcoma consists of preoperative chemotherapy, surgical tumor excision, and postoperative chemotherapy. Limb-saving surgery is feasible in most cases. Advances in osteosarcoma treatment have now achieved a 5-year survival rate of 60% to 90% for patients, and limb function after reconstruction continues to improve with time [3, 16, 30, 46, 47, 49].

Tsuchiya et al. developed a new approach using frozen autografts [48] to improve reconstruction after osteosarcoma resection. The tumor is resected with an adequate margin, and the resected specimen is immersed in liquid

nitrogen for 20 minutes to kill all tumor cells. After thawing, the specimen is returned to the original place with appropriate internal fixation to reconstruct the defect. Compared with heat-treated bones [8, 14], bone genetic proteins and native biomechanical structures are preserved after cryotreatment [53]. In one report limb function using the technique of Tsuchiya et al. was rated as excellent in 71.4% of patients, and good in 10.7%, as assessed by the functional evaluation system of Enneking [11]. Two studies suggest the approach enhanced bone formation when compared histologically with pasteurized bone and irradiated bone [43, 48]. Another advantage in reimplanting cryotreated tumor tissue is its effect on the immune system [50]: tumor tissue after cryoablation in situ provokes an immune reaction in patients with breast and prostate cancer [6, 8, 39]. Brewer et al. reported metastatic tumors sometimes disappear or shrink after in situ cryoablation of the primary tumor with liquid nitrogen [4]. The structure of tumor antigens is retained in frozen tumor, and leukocytes probably can recognize these antigens. Similar antitumor effects can be expected from our reconstructive procedure of reimplanting tumor-bearing bone after cryotreatment with liquid nitrogen.

Nishida et al. observed an inadequate antitumor effect after reimplantation of frozen tumor tissue alone [35]. However, the antitumor effect was enhanced by promoting nonspecific immune activation by intraperitoneal injection of OK-432, a substance extracted from alpha-Streptococcus pyogenes. This approach promotes inflammation and activation of dendritic cells (DCs) that initiate the specific antitumor effect [19]. This type of immunotherapy reportedly is effective for breast and prostate cancers [6, 8, 39]. Many groups have reported successful immunotherapy for osteosarcoma [5, 15, 18, 20, 22, 24, 25, 33, 34, 36, 42, 51, 52]. However, the ability to control metastatic lesions and local recurrence does not appear to be superior to other adjuvant treatments [2, 7, 13, 23, 29].

We therefore wondered whether combining cryotreatment and immunotherapy might enhance tumor response. We specifically determined whether: (1) antitumor immunity could be enhanced through activation and transfer of DCs combined with reimplantation of the cryotreated primary tumor, and (2) metastatic lesions could be prevented owing to the involvement of T lymphocytes in a murine osteosarcoma model (LM8).

Materials and Methods

Using a reported method to induce osteosarcoma [1, 35], we hypodermically implanted 1×10^6 LM8 cells (a murine osteosarcoma cell line) into the subcutaneous gluteal region of 80 female C3H mice, 6 to 8 weeks old. Tumors developed in all animals. Two weeks after inoculation, we surgically excised the tumors and cryotreated them with liquid nitrogen. We established the following six groups (Fig. 1): (1) the tumor was excised with wide margins 14 days after inoculation ($n = 15$); (2) the tumor was excised with wide margins 14 days after inoculation and bone marrow-derived DCs then were injected into the contralateral subcutaneous gluteal region without reimplantation of the cryotreated primary tumor twice a week ($n = 15$); (3) the tumor was excised with wide margins 14 days after inoculation and reimplanted after cryotreatment with liquid nitrogen into the contralateral gluteal region to evaluate for local recurrence from frozen tumor tissue ($n = 15$); (4) the tumor was excised 14 days after inoculation and reimplanted after cryotreatment into the contralateral gluteal region to evaluate for local recurrence, and DCs then were injected twice a week into this secondary site ($n = 15$); (5) the tumor was excised with wide margins 14 days after inoculation and DCs exposed to cryotreated tumor lysates were injected twice a week into the contralateral gluteal region without reimplantation of the cryotreated primary tumor ($n = 15$); and (6) the tumor was excised with wide margins 14 days after inoculation and reimplanted after the treatment with liquid nitrogen into the contralateral gluteal region to evaluate for local recurrence (same as Group 3) with the addition of DCs exposed to cryotreated tumor lysates injected twice a week ($n = 15$). We harvested tumor from 30 mice, and then the tumor was treated with liquid nitrogen to create the lysates. We presumed a systemic immune response would be induced by injecting DCs around the frozen tumor tissue. We microscopically determined the presence of metastases in the lungs 2 weeks after the tumor inoculation. We had previously confirmed the presence of pulmonary metastases in an additional 20 mice in a preliminary experiment in advance. We also confirmed that there were no viable cells after cryotreatment using liquid nitrogen, in agreement with a previous study [35]. We observed no recurrence of the tumor at the primary site of inoculation after excision. All experiments were performed under the guidelines for animal experiments as stipulated by the Kanazawa University Graduate School of Medical Science [37].

LM8 cells, derived from Dunn osteosarcoma, were provided by the Riken BioResource Center (Saitama, Japan). The cells were maintained in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 μg streptomycin per mL, and 100 units penicillin per mL and were cultured at 37°C in 5% CO_2. To establish local implantation of the tumor and subsequent lung metastasis, the LM8 cells (1×10^6) were suspended in 0.2 mL phosphate-buffered saline (PBS) and subcutaneously inoculated into the right

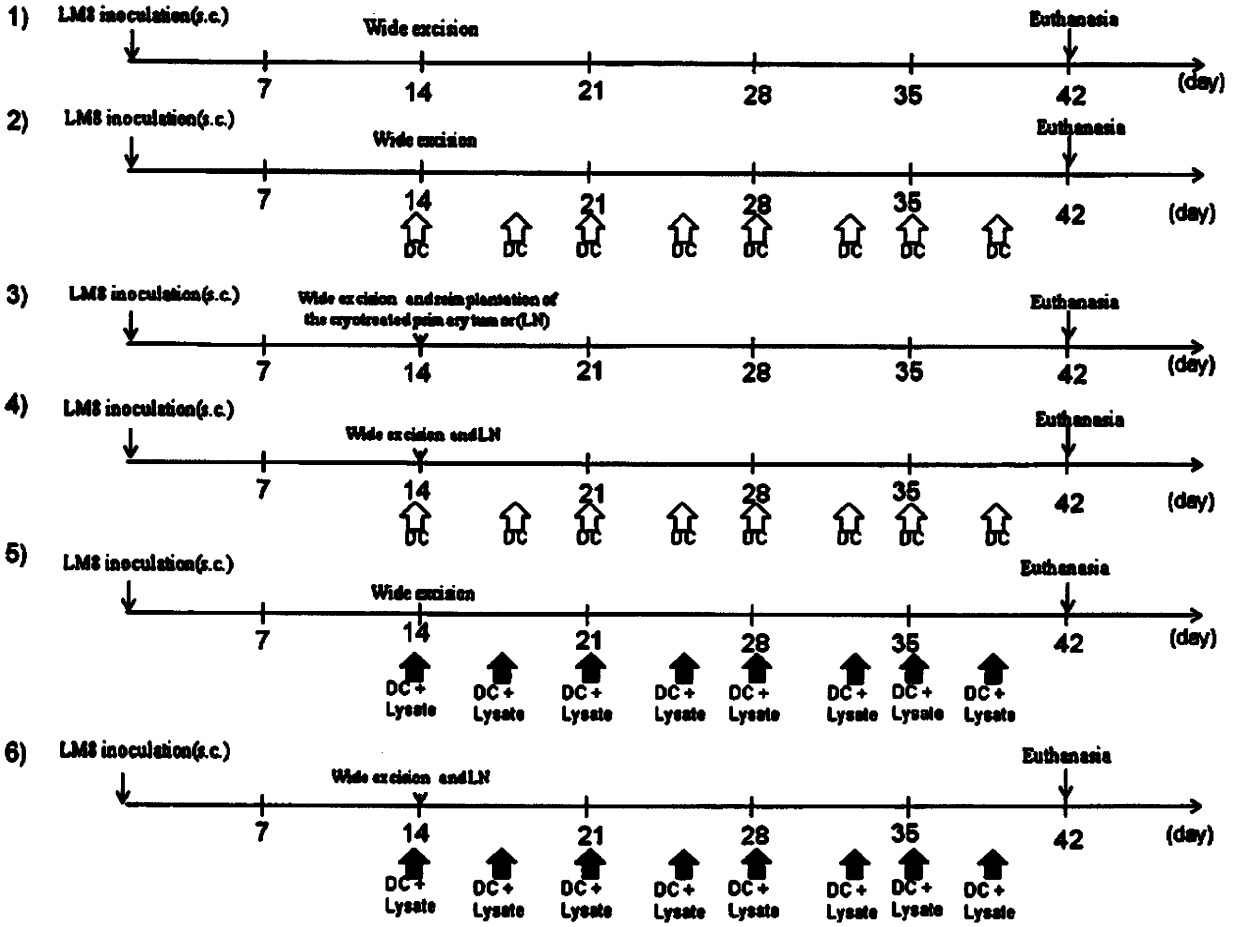


Fig. 1 A diagram of the experimental protocol and treatment schedule is shown. Two weeks after tumor inoculation, tumors were treated by one of the following methods: (1) excision only (n = 15); (2) DCs without reimplantation of the cryotreated primary tumor (n = 15); (3) reimplantation of the cryotreated primary tumor (n = 15); (4) DCs pulsed with cryotreated tumor lysates and reimplantation of the cryotreated primary tumor (n = 15); (5) DCs pulsed with cryotreated tumor lysates without reimplantation of the cryotreated primary tumor (n = 15); or (6) DCs pulsed with cryotreated tumor and reimplantation of the cryotreated primary tumor (LN) (n = 15). The mice were euthanized and evaluated 6 weeks after tumor inoculation. sc = subcutaneous.

gluteal region of the mice. All animals had macroscopically and microscopically confirmed lung metastases within 4 weeks [1].

C3H mice were purchased from Sankyo Labo Inc (Toyama, Japan) and housed in a specific pathogen-free animal facility in our laboratory. We were not able to accurately determine the survival time of each group because the guidelines for animal experiments concerning pain required euthanasia in distressed animals.

Liquid nitrogen (−196°C) was used for cryotreatment. Tumor tissue was collected on gauze and soaked in liquid nitrogen for 20 minutes for en bloc tumor tissue freezing. The tumor was prethawed at room temperature (20°C) for 15 minutes and then thawed in distilled water (20°C) for 15 minutes. The liquid nitrogen-treated tumor tissue was transplanted subcutaneously in the left gluteal region of the same mouse.

Because the mice were genetically identical, the structure of the major histocompatibility complex (MHC) Class I molecules was such that the T cells would be able to recognize the MHC Class I with antigens on the antigen-presenting cells (APCs) [17, 27]. Bone marrow-derived DCs were generated as described by Lutz and Rössner [28] with minor modifications. Briefly, erythrocyte-depleted mouse bone marrow cells obtained from flushed marrow cavities (1×10^6 cells/mL) were cultured in complete medium with 20 ng/mL recombinant mouse GMCSF (PeproTech EC Ltd, London, UK) in 10-cm tissue culture dishes at 37°C in an atmosphere containing 50 mL CO_2 per L. On Days 3 and 6, half of the medium was added to the

same volume of fresh complete medium and used to replenish the original plates. The freeze-thawed tumor lysate was added to the DC cultures on Day 6 at a ratio of five DC equivalents to one tumor cell (ie, 5:1) and incubated at 37°C in an atmosphere containing 50 mL CO_2 per L. After 24 hours of incubation, nonadherent cells including DCs were harvested by gentle pipetting.

For fluorescence activated cell sorting (FACS) analysis, DCs were counted with a FACSCalibur™ Flow Cytometer (Becton-Dickinson, San Jose, CA) and stained with fluorochrome-conjugated antibodies (BD Pharmingen, Tokyo, Japan) for the following markers: cluster of differentiation (CD)11c, CD80, CD86, I-Ad, and CD40. CD11c was used as a marker for all DCs regardless of the degree of maturation, whereas CD80, CD86, I-Ad, and CD40 are markers for DCs. Data analysis was performed with CELLQuest™ software (Becton-Dickinson). The corresponding labeled isotype antibodies served as controls. DCs used for vaccination were washed twice, enumerated, and resuspended in PBS at 1×10^6/mL.

We inoculated LM8 cells (5×10^6) in a mouse to make the tumor lysate. After 4 weeks, we resected the tumor mass and soaked the entire tumor in liquid nitrogen to kill the tumor cells. We mixed cryonecrotic tissue with DCs at Culture Day 6, after the tumor was defrosted, and the homogenate was prepared using PBS. The homogenate was passed through a 0.2-μm filter to remove bacteria and tissues and mixed with the DCs for 24 hours.

After intraperitoneal injection of 5 mL sodium pentobarbital (Somnopentyl®; Kyontsu Seiyaku, Tokyo, Japan), mice were euthanized by cervical dislocation and their blood was collected. Murine interferon (IFN)-γ and interleukin (IL)-4 release were measured by ELISA using Quantikine® (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol using an Easy Reader EAR340 microtest plate reader (SLT-Labinstruments, Salzburg, Austria).

We estimated the area of the pulmonary metastatic lesion on 50 serial histologic sections of each lung by manually drawing orthogonal lines delimiting the edges of the pulmonary metastatic lesion and selected the widest part of the specimen. The area was determined by multiplying the maximum orthogonal dimensions using ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). We compared the mean areas between the six groups.

For immunohistochemistry, lung specimens were fixed in 20% formalin and embedded in paraffin. For each case, we examined all the blocks of lung tissues of formalin-fixed, paraffin-embedded tumor tissue. All specimens were decalcified, although we found the decalcification step did not influence the immunohistochemistry for any of the stains. Five sections for each mouse were cut 4-μm thick. Each section was cut at the maximum diameter. CD8(+) T lymphocytes and natural killer (NK) cells in the pulmonary metastatic lesion were quantified by measuring the immunohistochemistry-positive cells per unit area in each group. Rehydrated tissue sections were incubated with rat monoclonal antibody raised against CD8(+) T lymphocytes of mouse origin (Santa Cruz Biotechnology, Santa Cruz, CA) and rat monoclonal antibody raised against NK cells of mouse origin (Abcam Plc, Cambridge, UK). The two antibodies were diluted 1:50 with PBS. Color reactions were performed at room temperature for 15 minutes and cover slips were mounted with glycerol and gelatin.

We determined differences in serum IFN-γ, serum IL-4, pulmonary metastatic area, and number of CD8(+) lymphocytes and NK cells in the metastatic area among the six groups using a nonrepeated-measures ANOVA and the Scheffe test. All analyses were conducted with SPSS® 11.0 software (SPSS Japan Inc, Tokyo, Japan).

Results

We activated antitumor immunity by combining DCs exposed to lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor. On Culture Day 7, the ratio of mature DCs to immature DCs was increased compared with the ratio at Culture Day 6 (Fig. 2; immature DCs, upper left; mature DCs, upper right). Moreover, this increase was more apparent in groups incubated with tumor lysate. Serum IFN-γ levels were greater ($p < 0.0001$) in the mice that received DCs combined with reimplantation of the cryotreated primary tumor (119.0 ± 7.61 pg/mL) than in the cryotreated primary tumor alone group (37.33 ± 2.58 pg/mL). Moreover, the group that received tumor lysate-exposed DCs combined with reimplantation of the cryotreated primary tumor (157.33 ± 14 pg/mL) had a greater ($p < 0.0001$) IFN-γ level than the group that received only tumor lysate-exposed DCs without reimplantation of the cryotreated primary tumor (120.27 ± 11.29 pg/mL) (Fig. 3). Serum IL-4 was lower ($p < 0.0001$) in the mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group (13.33 ± 9.75 pg/mL) than in the excision-only group (45.06 ± 5.71 pg/mL) (Fig. 4).

The enhanced immune response by T lymphocytes reduced metastatic lesions. Reduction of the metastatic area was greater ($p < 0.0001$) in the group that received DCs without reimplantation of the cryotreated primary tumor (15.99 ± 3.93 mm^2) than in the excision-only group (24.12 ± 3.60 mm^2). The reduction of the metastatic area was greater ($p < 0.0001$) in the DCs combined with reimplantation of the cryotreated primary tumor group (5.39 ± 1.49 mm^2) than in the reimplantation of

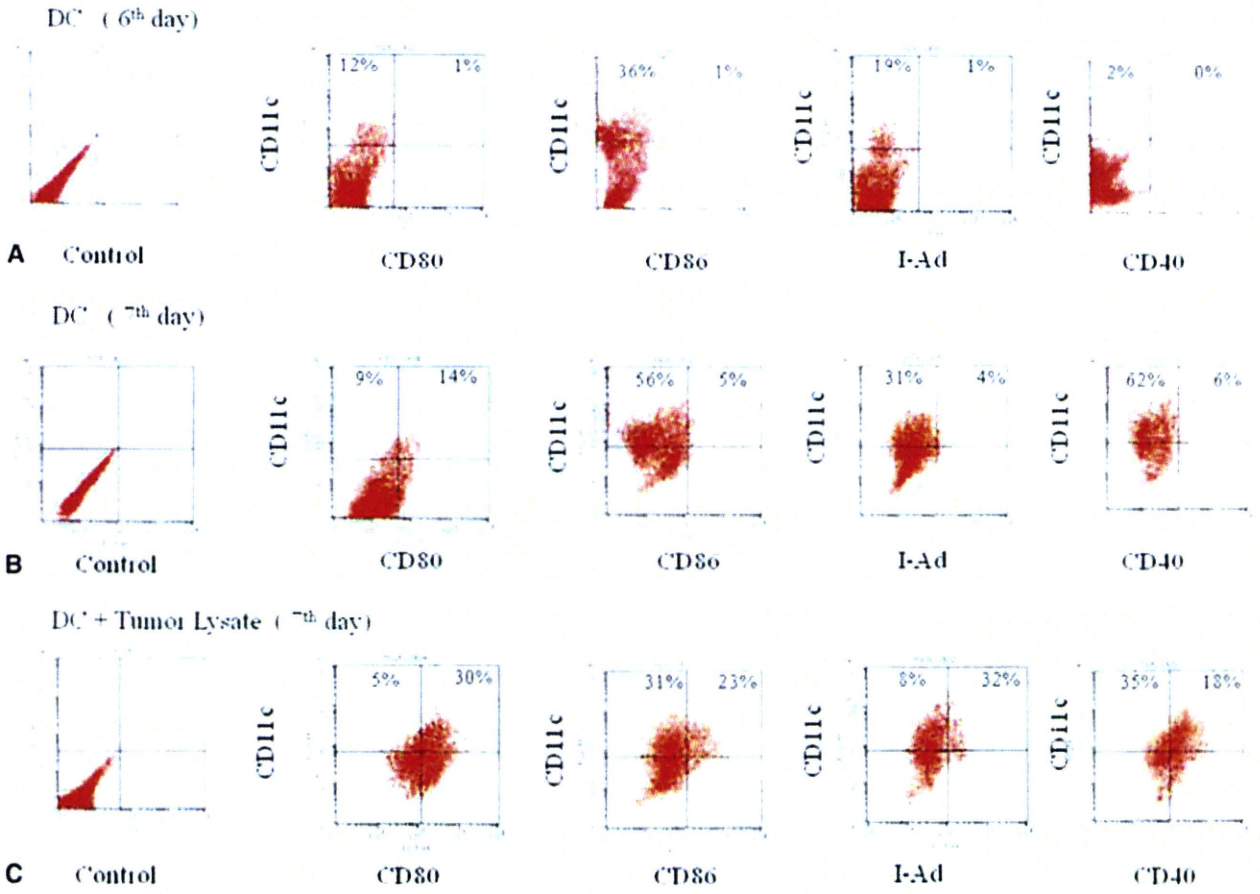
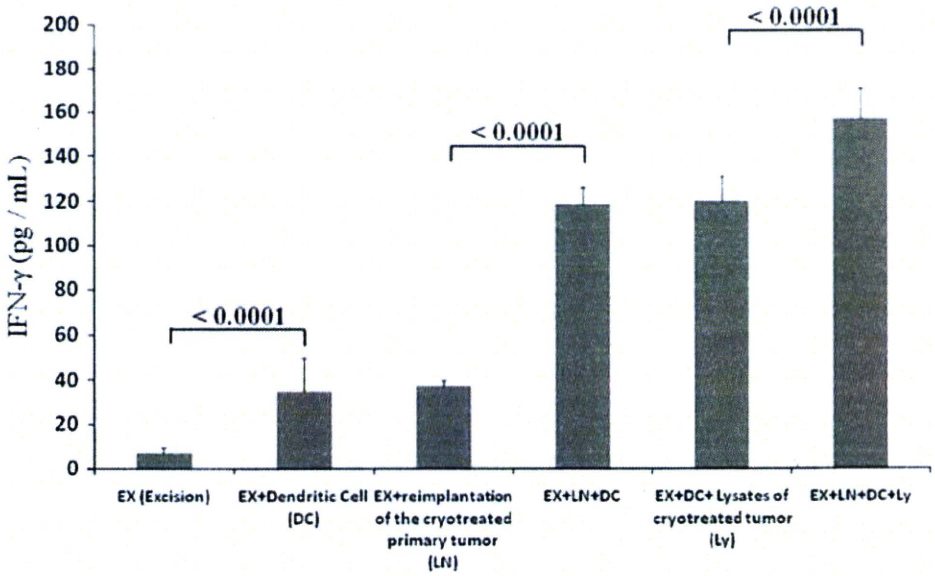


Fig. 2A–C DC activation status was examined using flow cytometry. DCs at Culture Day 7 (Group **B**) were more mature than DCs at Culture Day 6 (Group **A**). On Culture Day 7, DC maturity was greatest in the groups receiving lysate-primed DCs (Group **C**) than in those not receiving lysate-primed DCs (Group **B**).

Fig. 3 A graph of the serum IFN-γ levels in the six treatment groups is shown. The samples were collected 28 days after the reimplantation surgery and/or DC adoptive transfer. Mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a highest IFN-γ level. Error bars represent SD.



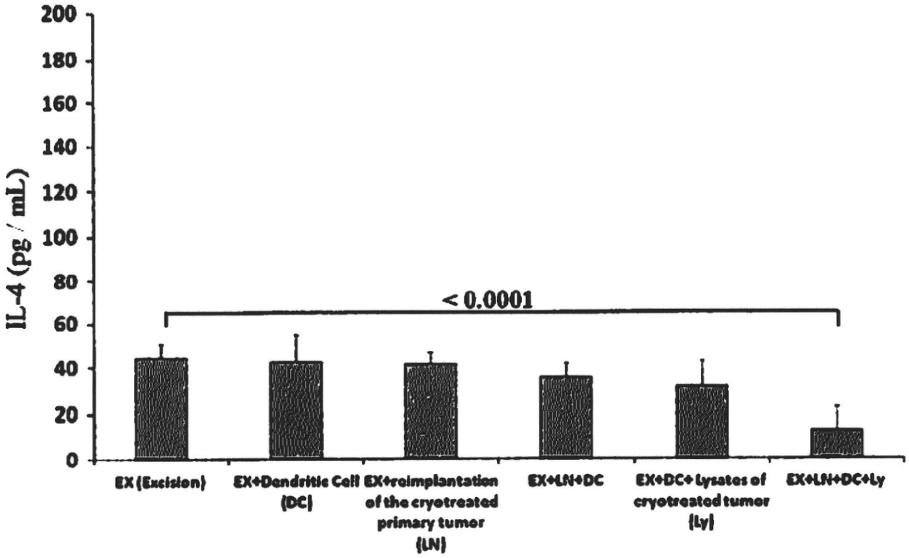


Fig. 4 A graph of the serum IL-4 in the six treatment groups is shown. Sera were collected 28 days after the reimplantation surgery and/or DC adoptive transfer. DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a lower level than any of the other groups. Error bars represent SD.

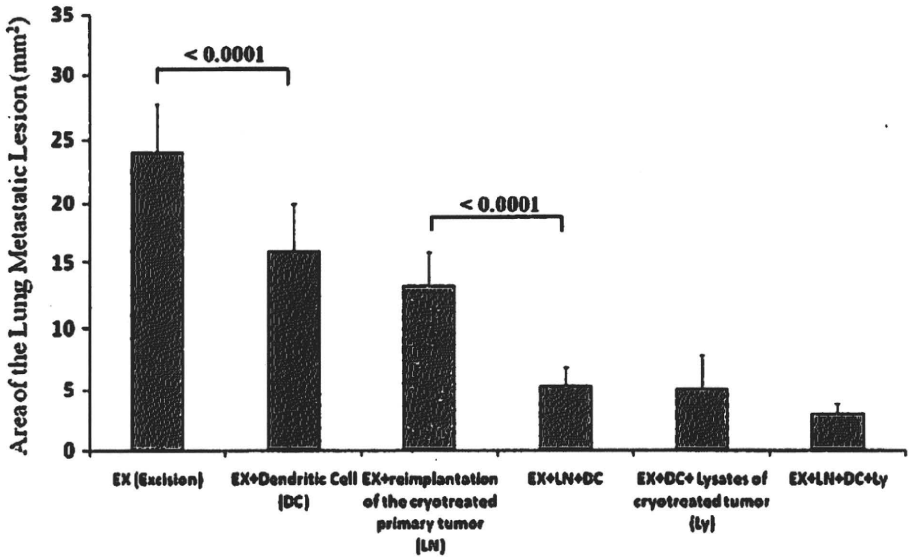


Fig. 5 Reduction of the metastatic area in the six treatment groups is shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. Error bars represent SD.

the cryotreated primary tumor alone group (13.22 ± 2.59 mm^2) (Fig. 5). CD8(+) T lymphocytes gathered in the pulmonary metastatic area in DC-treated groups, however, NK cells were not recruited to the metastatic area in the DC-treated groups compared with the nonDC-treated groups (Fig. 6). The number of CD8(+) T lymphocytes per unit area was greater ($p < 0.0001$) in the DCs combined with reimplantation of the cryotreated primary tumor group (8.33 ± 2.57 cells/mm^2) than in the reimplantation of the cryotreated primary tumor alone group (2.44 ± 0.53 cells/mm^2). Mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor (12.79 ± 2.14 cells/mm^2) showed higher ($p < 0.0001$) levels than the group that received DCs exposed to the lysates of cryotreated tumor without reimplantation of the cryotreated primary tumor (8.71 ± 2.39 cells/mm^2) (Fig. 7). The number of NK cells per unit area was greater ($p < 0.0001$) in the group that received DCs exposed to the lysates of cryotreated tumor without reimplantation of the cryotreated primary tumor (3.90 ± 2.17 cells/mm^2) than in the excision-only group (1.20 ± 0.30 cells/mm^2) (Fig. 8). The CD8(+)T lymphocyte, CD4(+) T lymphocyte, and DC infiltrations in reimplanted tumors were similar to those seen with pulmonary metastases (data not shown).

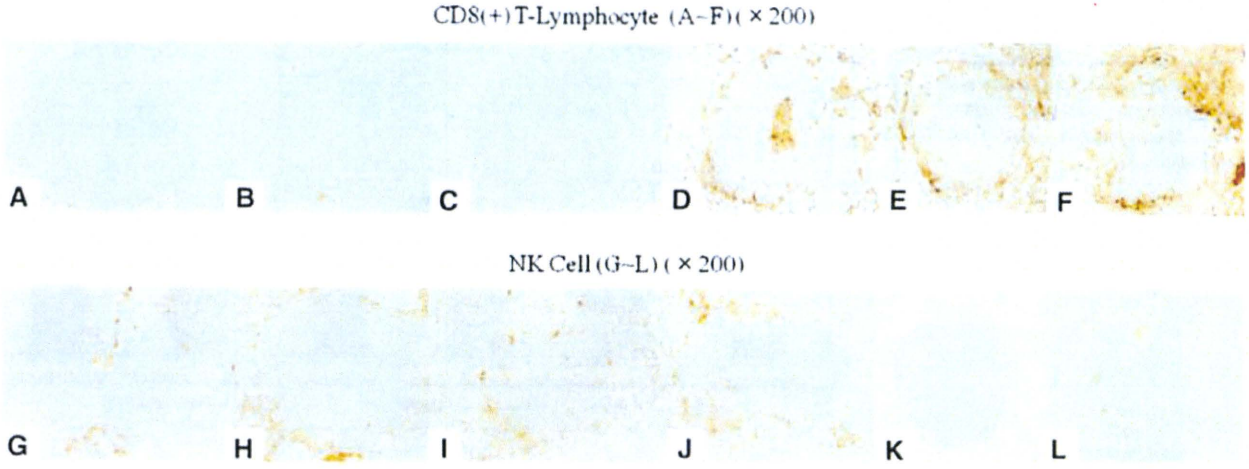
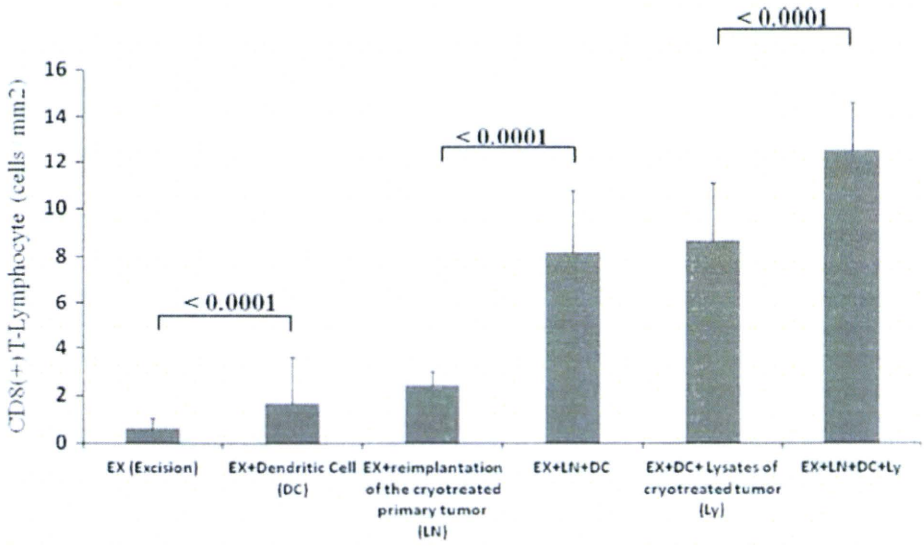


Fig. 6A–L To evaluate CD8(+) T lymphocytes and NK cells in pulmonary metastasis, immunostaining was performed: (**A**) CD8(+) T lymphocytes in Group 1, (**B**) CD8(+) T lymphocytes in Group 2, (**C**) CD8(+) T lymphocytes in Group 3, (**D**) CD8(+) T lymphocytes in Group 4, (**E**) CD8(+) T lymphocytes in Group 5, (**F**) CD8(+) T lymphocytes in Group 6, (**G**) NK cells in Group 1, (**H**) NK cells in Group 2, (**I**) NK cells in Group 3, (**J**) NK cells in Group 4, (**K**) NK cells in Group 5, and (**L**) NK cells in Group 6. CD8(+) T lymphocytes gathered in Groups D,E, and F. However, they did not gather in Groups A, B, and C. However, NK cells were recruited only in Groups A, B, and C. (Original magnification, ×200).

Fig. 7 The numbers of CD8(+) T lymphocytes per unit area in the six treatment groups are shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a higher level than any other groups. Error bars represent SD.



Discussion

Various immunotherapies for osteosarcoma have been tried. As standard treatments for osteosarcoma are ineffectual for many patients, new treatments need to be developed. In the 1970s, immunotherapy for osteosarcoma was reported by Southam et al. [42], Neff and Enneking [34], and Campbell et al. [5]. In the 1980s, new methods such as the use of interferons and Bacille de Calmette et Guérin were reported [22, 24, 36]. Another approach used antiidiotypic antibodies using T cells and liposome encapsulation [18, 51, 52]. Current methods of immunotherapy for osteosarcoma include peptide therapy or gene transfer therapy combined with hyperthermia therapy [10, 15, 21, 25, 33]. We asked whether (1) antitumor immunity could be achieved through activation of DCs combined with reimplantation of the cryotreated primary tumor and (2) if metastatic lesions would be prevented owing to enhanced T lymphocyte involvement.

We acknowledge limitations in this study. First, we used mice with an identical genetic makeup. The structure of the MHC Class I molecules was similar and the T cells could recognize the MHC Class I. However, we needed to use DCs from a different (albeit genetically identical) mouse to

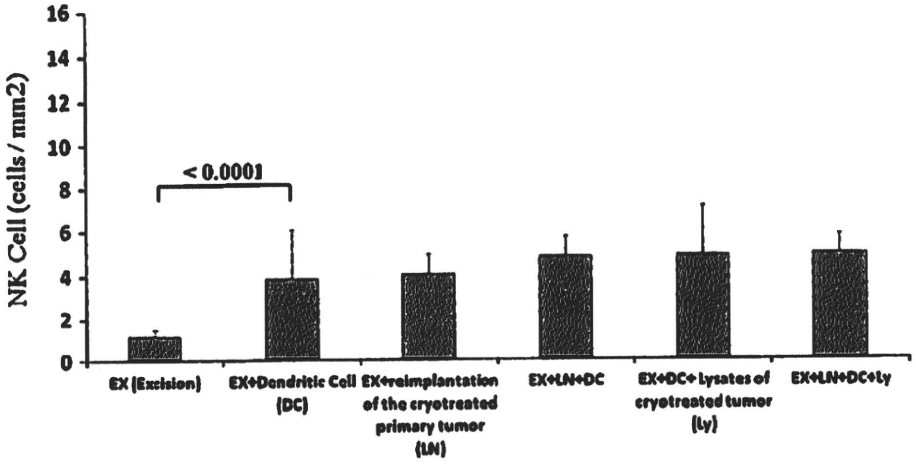


Fig. 8 The numbers of NK cells per unit area in the six treatment groups are shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. Error bars represent SD.

accomplish our adoptive transfer experiments. We minimized the potential for an immune response to nonself antigens by using genetically identical tumor tissue and mice. It would be necessary to use DCs derived from the same individual in clinical application, but this could not be achieved in our mouse model. In humans, however, monocytes are separated from the patient's own peripheral blood and DCs can be induced from these monocytes. Second, we could not completely replicate the clinical approach used in humans in our mouse model. In clinical cases frozen bone always is returned to the same site. However, it was impossible to replicate this in our experimental mouse model in which transplanted tumor cells were removed from the tibia and then returned to the same place after cryotreatment. In a preliminary experiment we attempted to do just that and these 20 mice could not move and died of starvation. We therefore used the contralateral gluteal region to check for local recurrence after tumor excision or recurrence from frozen tissue.

Antitumor immunity appeared to be activated through DCs combined with reimplantation of the cryotreated primary tumor or by exposing the transferred DC to lysates of cryotreated tumor. The use of lymphokine-activated killer (LAK) therapy has been used with other types of tumors [26]. However, T lymphocytes, which are the effectors, do not accumulate inside osteosarcoma tumors as expected. Autoclaving supplemented by DCs is thought to enhance the antitumor effect, but hyperthermia causes proteins to denature, and activation of the antitumor effect is often insufficient [37]. Several studies [12, 31, 41] report peptide vaccine therapy, but many patients apparently develop immunotolerance [45]. Thus, immunotherapy for malignant tumor achieved by these various methods has not been established definitively although investigations continue to try to overcome the major hurdles associated with immunotherapy (Table 1). We emphasize the immune response is activated by cryotreatment but not by heat-treated tissue. Our method differs from those described by others [7, 9, 10, 14]. In some regards DCs are believed to be the principal APCs for initiating immune responses in vivo [32]. In comparison with other traditional adjunct therapeutic options for cancer, such as radiation therapy and chemotherapy, immunotherapy provides a more targeted treatment to the cancer, with potentially fewer detrimental effects on noncancerous cells [30, 40]. DCs without sufficient cancer antigens may not have the ability to kill tumor cells and present the antigen to T lymphocytes by themselves. Our data suggest the antitumor effect in the group that received DCs without reimplantation of cryotreated primary tumor was almost the same as that in the reimplantation of cryotreated primary tumor alone group. The data further suggest the effects increased only when exposing the DCs to tumor lysates in the absence of cryonecrotic primary tumors. However, combining reimplantation of cryotreated primary tumor and DCs exposed to cryotreated tumor lysates produced synergistic effects. Using reimplantation of cryotreated primary tumor is more appropriate for clinical applications. We therefore believe an efficient immune response will be activated when DCs recognize tumor antigens appropriately. CD8(+) T cells act as an effector by the Th1 route, and this is promoted mainly by IFN-γ and IL-12 [38]. However, IL-4 [21], IL-6, and IL-10 strengthen humoral immunity. Levels of IFN-γ, IL-2, and IL-12 generally increase when cell-mediated immunity is activated, and IL-4, IL-6, and IL-10 increase when humoral immunity is activated. These cytokines act in opposition to maintain an immune balance.

Our data suggest enhanced T lymphocyte recruitment and function reduce metastatic lesions in a murine osteosarcoma model. Immunoreactivity increased slightly in mice that received DCs exposed to lysates of cryotreated tumor combined with reimplantation of the cryotreated primary tumor. NK cells attack the tumor independently of APCs. NK cells attack cells that downregulate MHC Class

Table 1. Immunotherapeutic trials of malignant tumors

Tumor	Immune intervention	Route	Immunologic response	Comments	References
Osteosarcoma	BCG	SC	NC	No consistent clinical effect	[22, 24]
Osteosarcoma	Interferon α	SC, IV	PR–NC	Osteosarcoma-associated antigens have potential for targeted immunotherapy	[36]
Unknown	LAK	IV	NC	T lymphocytes were unable to penetrate the tumor	[26]
Osteosarcoma	Antiidiotypic antibodies	IV	NC	It may be possible to circumvent this heterogeneity by activation of tissue macrophages to the tumoricidal state	[18, 51, 52]
Breast cancer, osteosarcoma	Peptide therapy combined with hyperthermia therapy	SC, IV	NC	It may be a potential agent for use in immunotherapy	[15, 20]
Osteosarcoma	Gene transfer therapy combined with hyperthermia therapy	IV	NC	IL-23 seems to be a less effective immunotherapeutic for adjuvant treatment of osteosarcomas	[25, 33]
Unknown	Peptide vaccine therapy	SC	NC–PD	Many patients have peptide-induced tolerance develop	[45]
Osteosarcoma	Cryoimmunology and DCs	SC	PR	Combining cryotreatment with DCs resulted in enhanced antitumor effects	Our data

BCG = Bacille de Calmette et Guérin; SC = subcutaneous; NC = no change; IV = intravenous; PR = partial response; LAK = lymphokine-activated killer; IL = interleukin; PD = progressive disease; DCs = dendritic cells.

I expression or have a stressed appearance [44]. We observed a reduced tumor burden in the groups that received transplanted DCs, which correlated with recruitment of CD8 lymphocytes to the tumor site as observed with immunohistochemistry.

Returning the frozen bone after liquid nitrogen treatment to its original place can be readily used in the clinic. After the first cryotreatment, it is possible to perform the treatment again using cultured DCs if a patient's tumor cells have been preserved. This approach therefore still can be used even after other methods (such as chemotherapy, radiation therapy, or surgery) no longer are reasonable. Combining DCs pulsed with lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor enhanced antitumor effects. We believe the approach may be a useful alternative for patients with osteosarcoma when other treatment options including chemotherapy, radiotherapy, and surgical treatment have been ineffective.

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