Prognostic Significance of Adoptive Immunotherapy with Tumor-associated Lymphocytes in Patients with Advanced Gastric Cancer: A Randomized Trial[1]

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ABSTRACT

Purpose: **We performed adoptive immunotherapy (AIT) with tumor-associated lymphocytes (TALs) in combination with chemotherapy in patients with advanced-stage gastric cancer in a randomized controlled study and investigated whether or not an improved survival effect is observed with AIT.**

Experimental Design: **Forty-four consecutive patients with stage IV gastric cancer [staged according to the International Union against Cancer classification] were prospectively assigned to the control group (chemotherapy alone) or the AIT group (AIT plus chemotherapy). Patients in the AIT group received an adoptive transfer of cultured TALs in combination with low-dose cisplatinum/5-fluorouracil chemotherapy, whereas patients in the control group received chemotherapy alone.**

Results: **The 50% survival rates were 11.5 and 8.3 months in the AIT and control groups, respectively. The overall survival of patients in the AIT group was significantly better than that of patients in the control group, as analyzed by the log-rank test ($P < 0.05$). Multivariate analysis with Cox's proportional hazards model revealed that AIT provided an independent prognostic factor, indicating that AIT influenced patient survival in a positive manner.**

Conclusions: **AIT with TALs in combination with chemotherapy was effective in prolonging survival in patients with stage IV gastric cancer.**

INTRODUCTION

Gastric cancer is one of the most common cancers in Japan today. Despite various treatments such as surgery combined with chemotherapy (1), hyperthermia (2), or chemoradiotherapy (3), control of this cancer at the advanced stage remains difficult. The possibility of applying immunotherapy for this type of cancer would therefore be highly desirable. We and others have reported that CTLs from gastric cancer patients, which are restricted to MHC class I, can react specifically against autologous tumor cells (4, 5). Therefore, AIT[3] with tumor-specific CTLs represents an alternative approach for treatment of patients with gastric cancer.

It has been reported that the adoptive transfer of tumor-infiltrating lymphocytes in combination with IL-2 to patients with advanced-stage lung cancer (6), malignant melanoma, and renal cell carcinoma (7) resulted in prolonged survival or a therapeutic effect. Tumor-specific CTLs, when adoptively transferred, have therapeutic activity and can induce regression of established tumors or micrometastasis (8). With respect to gastric cancer, it has been shown that AIT, when combined with the biological response modifier OK-432, had a survival effect for patients with gastric cancer and peritoneal metastasis (9). Yamaue *et al.* (10) reported that AIT by activated killer cells in combination with chemotherapy resulted in a clinical response in patients with advanced gastric cancer. Furthermore, we have reported that AIT with TALs had the effect of immunological modification in patients with gastric and colon cancers (11).

In the present study, we performed AIT with TALs in combination with chemotherapy in patients with advanced-stage gastric cancer in a randomized controlled study and investigated whether or not an improved survival effect is observed with AIT.

PATIENTS AND METHODS

Patients and Clinical Evaluation. Patients were eligible if they had histologically confirmed stage IV gastric cancer (staged according to the UICC classification), adequate bone marrow and renal function, and were <80 years old. One exclusion criterion was the presence of liver metastasis. Eligible patients were randomly assigned to the control group (chemotherapy alone) or the AIT group (AIT plus chemotherapy), on receipt of stage IV confirmation, no later than 10 days after surgery. All patients were monitored clinically using imaging analysis such as ultrasonography and computed tomography and classified according to conventional criteria: (*a*) complete response was defined as the disappearance of all measurable tumor for at least 1 month; (*b*) partial response was defined as

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[3] The abbreviations used are: AIT, adoptive immunotherapy; TAL, tumor-associated lymphocyte; IL, interleukin; UICC, International Union against Cancer; MR, minor response; NC, no change; PD, progressive disease; TNF-α, tumor necrosis factor α.

a ≧50% decrease of all measurable tumor for at least 1 month; (*c*) MR was defined as a reduction of 25–49%; (*d*) NC was defined as a reduction of <25%; and (*e*) PD was defined as an increase in the tumor burden. Cytological examination of ascites and pleural effusions were performed periodically, and the cells were diagnosed as class I to class V according to the Papanicolaou classification. The present study was approved by the ethical committee of Yamanashi Medical University, and written informed consent was obtained from all individuals.

Treatment Design. All patients in both groups underwent surgery. Then, from the 5th week after surgery for 5 months, all patients received chemotherapy with cis-platinum (10 mg/day) plus 5-fluorouracil (500 mg/day) by venous infusion once a week and oral doxifluridine (600 mg/day) daily. In the AIT group (chemotherapy plus AIT), TALs were cultured for 6 weeks, and the cultured TALs were administered by i.p. bolus injection to autologous donors with malignant ascites or by i.v. bolus injection to autologous donors with malignant pleural effusion or lymph node metastasis.

All adverse events were evaluated according to the WHO toxicity scale, and treatment was stopped if toxic effects of grade 3 or 4 developed.

Preparation of Lymphocytes and Tumor Cells. A single-cell suspension was prepared from malignant ascites or pleural effusions by 100% Ficoll (Pharmacia, Uppsala, Sweden) separation. The suspension was prepared from lymph node metastases obtained at surgery by mechanical mincing. Tumor- and lymphocyte-enriched populations were prepared by centrifugation of the single-cell suspensions through a discontinuous 75%/100% Ficoll gradient (5, 11, 12).

Generation of TAL Lines. To generate tumor cell lines, tumor-enriched populations from ascites, pleural effusions, or lymph node metastases in the patients were incubated for 60 min at 37°C to remove macrophages and fibroblasts by adherence to the plastic surface. Nonadherent cells were then cultured in RPMI 1640 (Life Technologies, Inc.) with 10–20% FCS, 50 units/ml penicillin, and 2 mM L-glutamine (Sigma, St. Louis, MO). Freshly separated or early-passage cultured tumor cells were frozen in aliquots or used fresh when available as feeders or stimulators in a TNF-α release assay.

TALs were generated by culturing the lymphocyte-enriched population using repetitive stimulation with an autologous tumor. The TALs (1–2 × 10^6 cells/ml) were kept in AIM-V medium (Life Technologies, Inc.) supplemented with 50 IU/ml recombinant IL-2 (Shionogi) by stimulation with autologous tumor treated with mitomycin C (50 μg/ml; Kyowa Hakkou, Japan) every 2 weeks at a ratio of 10–20:1 (lymphocyte:tumor), as described previously (5, 11, 12).

Characterization of Cultured TALs. The phenotypes of cultured TALs were analyzed by flow cytometry using CD3, CD4, CD8, and CD16 monoclonal antibodies (Becton Dickinson, Mountain View, CA) after 6 weeks of culture. To study the reactivity of cultured TALs against autologous tumor cells or allogeneic gastric cancers, a TNF-α release assay was performed after 6 weeks of culture. Briefly, mixtures of 1 × 10^5 effector cells (TALs) and 1 × 10^5 stimulator cells (tumor cells) were cultured in AIM-V medium with recombinant IL-2 (50 IU/ml) at 37°C for 12 h. Autologous tumor cells and allogeneic gastric cancer cell lines (MKN28 and MKN45) were used as stimulators. The supernatants of the cultured mixtures were collected, and the TNF-α content was determined using an ELISA kit (Immunotech, Marseille, France) according to the manufacturer's recommendations. When the amount of TNF-α for the autologous tumor cells was more than 2 times that for the allogeneic tumor cells, the reactivity was evaluated as preferential for autologous tumor cells.

Table 1 Clinicopathological factors of patients with stage IV gastric cancer[a]

	Control group (n = 22)	AIT group (n = 22)
Age (yrs)	66.2 ± 10.3	65.9 ± 10.1
Sex (male:female)	9:13	8:14
Tumor size (cm)	5.5 ± 2.1	5.0 ± 2.2
Depth of tumor invasion[a]		
T_1	0	0
T_2	0	0
T_3	18	17
T_4	4	5
Peritoneal dissemination		
−	4	4
+	18	18
Lymph node metastasis[a]		
N_0	0	0
N_1	5	4
N_2	17	18
Distant metastasis		
−	20	21
+	2	1

[a] Stage and clinicopathological factors were evaluated according to the tumor-node-metastasis (TNM) classification of the UICC.

Statistics. Differences between groups were compared using the Mann-Whitney test (nonparametric statistical analysis) or χ^2 test and considered statistically significant when *P*s were <0.05. The cumulative survival curve was estimated by the Kaplan-Meier method, and the differences in this distribution were compared with the log-rank test. Multivariate analysis of prognostic factors for overall survival was performed using Cox's proportional hazards model. All analysis was performed with intention to treat. All time estimates were made with the date of surgery as the baseline.

RESULTS

Patient Groups. Forty-four patients were randomly assigned to the control group (chemotherapy alone) or the AIT group (AIT plus chemotherapy) after surgery. There were no differences in the clinicopathological findings between the two groups, as shown in Table 1. Furthermore, there were no differences with regard to the extent of surgery performed or the extent of residual tumor (Table 2).

Three patients in the AIT group were not able to complete the treatment protocol because TALs could not be maintained in culture for two of them (patients 6 and 11) and one patient refused any treatments (patient 13), as shown in Table 3. These three patients were included in the analysis of the AIT group because an intent to treat analysis was performed. Thus, 22 patients in the control group and 22 patients in the AIT group were evaluated in this study.

Table 2 Background surgery before treatment

	Control group ($n = 22$)	AIT group ($n = 22$)
Gastrectomy		
+	20	21
−	2	1
Lymph node dissection		
+	20	21
−	2	1
Residual tumor[a]		
R_0	0	0
R_1	4	3
R_2	18	19
Operation time (min)	232 ± 159	221 ± 139

[a] Residual tumor status after surgery was evaluated according to the tumor-node-metastasis (TMN) classification of the UICC. R_0, no residual tumor; R_1, microscopic residual tumor; R_2, macroscopic residual tumor.

Clinical Outcome after AIT. When the cultured autologous TALs were transferred to the patients, no complications, with the exception of low-grade fever, were observed.

Of the 22 patients in the AIT group, 2 patients showed a MR with diminished ascites or pleural effusion and negativity of cytology in the fluid, 5 showed NC, and 15 showed PD (Table 3). In the control group ($n = 22$), 1 patient showed a MR, 4 revealed NC, and 17 revealed PD. The cause of death in all patients in the both groups was disease progression of gastric cancer.

The number of transferred TALs reached 9×10^8 to 5×10^9, and most of the transferred T cells were CD8+-dominant TAL lines (Table 3). Preferential reactivity to the autologous tumor cells was observed in 3 (patients 1, 5, and 14) of 12 TAL lines tested. When the amount of TNF-α for autologous tumor cells was more than 2 times that for allogeneic tumor cells, the reactivity was evaluated as preferential for autologous tumor cells. There was no clear correlation between clinical response and preferential reactivity to autologous tumor cells (Table 3). However, the amount of TNF-α for autologous tumor cells in patients with a MR or NC was significantly higher than that in patients with PD in the AIT group ($P < 0.05$; Table 3).

In addition, the survival time in the AIT group did not correlate with the number of transferred cells, the amount of TNF-α released, or clinical response (Table 3).

Survival Effect of AIT. Cumulative survival rates were compared in two groups, as shown in Fig. 1. The 50% survival rates were 11.5 and 8.3 months in the AIT group and control group, respectively. The overall survival of the patients in the AIT group was significantly better than that of the patients in the control group, as analyzed by the log-rank test ($P < 0.05$).

To assess whether AIT represented a prognostic parameter, we used Cox's proportional hazards model. The covariate parameters included age, gender, tumor size, depth of tumor invasion, peritoneal dissemination, lymph node metastasis, gastrectomy, and AIT. Multivariate analysis revealed that tumor size and AIT were independent prognostic factors (Table 4), indicating that AIT influenced patient survival in a positive manner.

DISCUSSION

In the present study, we showed that AIT with TALs in combination with chemotherapy prolonged the survival of patients with advanced-stage gastric cancer in a randomized controlled study.

AIT with lymphokine-activated killer cells was previously reported to have a therapeutic clinical effect in the treatment of melanoma or renal cell carcinoma (13, 14). However, it has also been shown that lymphokine-activated killer cell therapy was not effective in other types of cancer (15, 16) or for patients with advanced-stage disease (17, 18). Recently, adoptive transfer of activated T cells by stimulation with CD3 plus IL-2 was reported to be effective as an adjuvant treatment of hepatocellular carcinoma (19). Thus, to enhance the immune response against the tumor, we generated activated T-cell lines derived from TALs, and the TALs were adoptively transferred to the patients in combination with chemotherapy.

Interestingly, overall survival was significantly improved by AIT in comparison with the control group, even if we did not obtain clear clinical responses such as a complete response or even a partial response in the patients treated with AIT. This observation indicates that the transferred TALs play a functional role, to some extent, in the modulation of antitumor immunity, resulting in prolonged survival even if most of the patients received nonspecific T cells. In fact, the amount of TNF-α for autologous tumor cells in patients with MR or NC was significantly higher than that in patients with PD in the AIT group. It is possible that the transferred TALs recognized tumor cells and had the ability to produce TNF-α as the antitumor immune response, regardless of specific or nonspecific recognition. In addition, we have recently shown that AIT with TALs induced immunomodulatory effects such as an increase or a stabilization in the expression of signal-transducing TCR ζ molecules (11), and some of these patients were included in the present study.

In addition, most of the treated patients received adoptive transfer via the i.p. route in the present study. It has been shown that the nonspecific biological response modifier OK-432 has clinical effects relating to improvement of host immunity and prolongation of survival in peritoneal metastasis of gastric cancer (9, 20). OK-432, a streptococcal preparation, has the ability to induce cytokine production from macrophages or neutrophils in a nonspecific manner and to induce an inflammatory reaction inside the peritoneal space (20). Thus, it is also speculated that adoptively transferred TAL cells induced a nonspecific inflammatory reaction inside the peritoneal space, and the inflammatory reaction may be related to the improvement in survival.

However, several difficulties still remain to be resolved in AIT, especially in patients with gastric cancer. First, the selection and generation of an effective lymphocyte population for adoptive transfer are poorly understood. In previous clinical trials, tumor regression was assumed to reflect direct tumor lysis mediated by infused T cells, especially CD8+ CTLs (21). However, it was also suggested that adoptive transfer with a combination of CD4+ and CD8+ T cells, rather than CD8+ T cells only, was important because cytokines derived from CD4+ T cells were required for the induction and maintenance of an antitumor response (22). In the present study, we showed that the amount of TNF-α for autologous tumor cells in patients

Table 3 Characteristics of patients treated with adoptive transfer of cultured TALs[a]

Patient no.	Age (yrs)/Sex	PS	Origin of TALs	No. of transferred cells ($\times 10^9$)	% Positive cells in transferred cells				TNF-α release (pg/ml)			Route	Clinical response	Survival time (days)
					CD3	CD4	CD8	CD16	Auto	MKN28	MKN45			
1	50/F	1	As	4.8	97.9	19.0	78.2	3.9	456	102	23	IP	NC	661
2	79/F	1	As	4.0	98.1	12.3	86.8	2.0	289	246	42	IP	MR[b]	621
3	56/F	1	As	1.0	97.3	15.2	82.1	3.0	ND	12	25	IP	PD	347
4	72/F	2	As	2.1	97.3	49.0	48.9	4.6	56	24	34	IP	PD	280
5	76/F	2	As	0.9	90.1	26.9	62.7	8.9	220	98	67	IP	MR[c]	192
6	68/F	1											PD	363
7	71/M	1	Pl	0.9	96.4	41.1	44.5	3.5	ND	55	21	IV	PD	345
8	79/F	1	As	0.9	97.3	24.5	73.6	2.1	22	10	10	IP	PD	787
9	50/M	1	As	1.2	98.1	33.9	61.4	1.1	ND	98	111	IP	PD	439
10	67/F	1	As	4.6	89.9	44.5	45.2	9.3	23	34	23	IP	PD	451
11	61/F	2											PD	190
12	70/M	2	As	3.6	91.1	23.4	77.2	8.9	ND	75	22	IP	NC	154
13	71/F	1											PD	367
14	49/F	1	As	2.5	95.3	45.3	52.1	2.3	321	100	122	IP	PD	429
15	51/F	2	LN	1.0	96.1	34.1	60.1	4.9	ND	51	12	IV	NC	388
16	72/M	1	LN	2.0	92.1	21.5	70.1	5.9	52	22	14	IV	PD	621
17	72/F	1	As	3.0	96.5	19.1	78.2	2.5	215	116	45	IP	NC	664
18	71/M	1	As	1.2	91.5	42.1	50.1	0.9	ND	120	55	IP	PD	367
19	70/F	1	As	3.5	97.1	22.5	71.0	4.5	ND	251	151	IP	PD	395
20	51/M	2	LN	4.5	91.1	35.1	56.1	8.9	59	71	55	IV	PD	160
21	78/M	1	As	5.1	91.5	22.1	67.1	9.0	145	121	198	IP	NC	283
22	65/M	1	As	4.1	98.1	40.1	57.4	1.9	21	45	18	IP	PD	369

[a] Except for patients 6, 11, and 13, patients with gastric cancer were treated with adoptive transfer of TALs via i.p. (IP) or i.v. (IV) infusion. Autologous TALs isolated from malignant ascites (As), pleural effusions (Pl), or metastatic lymph nodes (LN) were expanded by repeated stimulation with autologous tumor cells in low-dose recombinant IL-2. The phenotypes of cultured TALs were analyzed by flow cytometry. To study the reactivity of cultured TALs against autologous tumor cells (Auto) and allogeneic gastric cancers (MKN28 and MKN45), TNF-α release assay from TALs was performed. F, female; M, male; PS, performance status; ND, not determined.

[b] Diminished ascites, class V→I.

[c] Diminished ascites, class V→II.

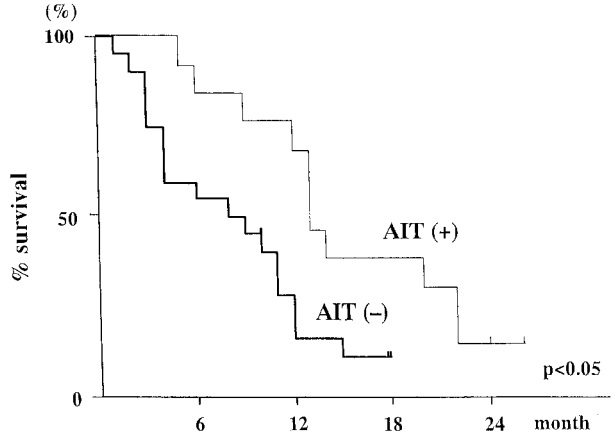

Fig. 1 Cumulative survival rate after AIT. The cumulative survival curve was estimated by the Kaplan-Meier method, and the differences in the distributions were compared by the log-rank test.

Table 4 Association of clinicopathologic factors with overall survival evaluated by Cox's proportional hazards model

	Significance (*P*)	Hazard ratio
Age[a]	0.800	0.921
Sex (male *vs.* female)	0.651	1.123
Tumor size[a]	0.027	1.113
Depth of tumor invasion (T_1–T_4)[b]	0.165	1.155
Peritoneal dissemination (p_0–p_3)[c]	0.071	1.315
Lymph node metastasis (N_0–N_3)[d]	0.812	1.229
Gastrectomy	0.261	0.900
Adoptive immunotherapy	0.048	0.819

[a] Continuous variables.

[b] T_1, tumor invades lamina propria or submucosa; T_2, tumor invades muscularis propria or subserosa; T_3, tumor penetrates the serosa; T_4, exposed serosa tumor invades adjacent structures.

[c] P_0, no peritoneal metastasis; P_1, metastasis to the adjacent peritoneum but not the distant peritoneum; P_2, some metastasis to the distant peritoneum; P_3, numerous sites of metastasis to the distant peritoneum.

[d] N_0, no evidence of lymph node metastasis; N_1, N_2, and N_3, metastasis to lymph nodes of groups 1, 2, and 3, respectively.

with MR or NC was significantly higher than that in patients with PD in the AIT group. This observation indicates that the tumor's ability to produce cytokine may be a surrogate maker that predicts responsiveness to AIT, although the amount of cytokine released did not correlate with survival time. Secondly, even if the effective CTLs are transferred adoptively to the patients, there are several mechanisms by which tumor cells can escape from tumor-specific T-cell surveillance in the tumor microenvironment *in vivo* (23, 24). The presence of factors such as Fas-Fas ligand interaction, oxidative metabolites, or immunosuppressive cytokines can be predicted to rapidly shut off the effector functions of CTLs (23, 24). In fact, preferential reactivity for autologous tumor cells was observed in 3 of 12 TAL

cell lines tested. However, there was no clear correlation between clinical response and reactivity to the autologous tumor cells. It is speculated that there are several mechanisms by which tumor cells can escape from CTLs, even if the specific CTLs are transferred to the patients.

In conclusion, AIT with TALs in combination with chemotherapy was effective in prolongation of survival in patients with stage IV gastric cancer. Furthermore, we have recently identified HER-2/neu-derived peptides as CTL epitopes recognized by gastric cancer-specific CTLs (12). Thus, AIT based on gastric cancer-specific CTLs or vaccination based on peptide epitopes may represent new modes of therapy for gastric cancer. Elucidation of the mechanisms of tumor escape from the immune response is important to the development of novel AIT and vaccine strategies against gastric cancer.

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