Adoptive immunotherapy using autologous lymphocytes activated *ex vivo* with antigen stimulation for patients with incurable cancer

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ABSTRACT Adoptive immunotherapy (AIT) using autologous lymphocytes activated *ex vivo* with antigen stimulation, including zoledronate-activated killer (ZAK) cells, were conducted in the treatment of patients with incurable cancer. Efficacy, safety and treatment feasibility were all evaluated, retrospectively. Two-hundred and twenty-eight patients were enrolled and 198 were treated with AIT every 3 weeks. Success of effector cell generation was evident in 94.0 % of the culture. A mean number of the administration was 6.8 times with a total number of 5.8 x 10^9 cells. Survival analysis implied marginal benefit of AIT in addition to chemotherapy in lung, colorectal, pancreatic cancers, especially in biliary cancer, showing the median survival time of 11.9 months. Objective tumor response of 3 CR and 6 PR was observed in colorectal, pancreatic, breast and biliary cancers, showing a response rate of 13.2 %. Improvement of QOL was replied in 33 % patients and FACT-BRM analysis demonstrated significant improvements in physical, social, emotional and functional well-beings. Together, it is suggested that AIT using autologous lymphocytes activated *ex vivo* with antigen stimulation, including ZAK cells, is safe and feasible, and may be effective in prolonging survival and improving QOL for patients with incurable cancer.

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INTRODUCTION

The discovery and molecular cloning of the crucial lymphocyte growth factor, interleukin-2 (IL-2), has facilitated the clinical application of adoptive immunotherapy (AIT) for cancer treatment using autologous lymphocytes activated *in vitro* with IL-2 [1]. We carried out *ex vivo* cell therapy for cancer treatment using activated autologous lymphocytes, including lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs), and tumor-sensitized lymphocytes, but tumor responses are limited with regard to quality of life (QOL) in locoregional administration for malignant effusion from gastrointestinal cancers [2]. Other researchers have demonstrated survival benefits in hepatocellular carcinoma patients using

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postoperative LAK cell transfer after surgery [3] and in lung cancer patients using LAK cell transfer in combination of chemoradiotherapy [4]. Thereafter, we introduced the use of dendritic cells (DCs) and tumor antigens into the effector cell generation system, although tumor responses were still limited [5].

Zoledronate, a bisphosphonate widely used to treat bone diseases, have been described to stimulate anti-tumor effector lymphocytes with $\gamma\delta$-type T cell receptors in the presence of DCs [6]. $\gamma\delta$T cells have been described to contain the properties associated with the innate immune system, HLA-unrestricted tumor recognition manner, high cytotoxic and proliferative potentials [7]. Sakamoto *et al*.[8] and Noguchi *et al*.[9] reported the AIT trial using zoledronate-activated lymphocytes for patients with cancer and described its safety and feasible profiles, indicating that AIT of cancer using zoledronate-activated lymphocytes may be highly promising for clinical use. We have previously established the generation system of zoledronate-activated killer (ZAK) cells [10]. In the present study, we conducted an observation study of AIT using autologous lymphocytes activated *ex vivo* with antigen stimulation, including ZAK cells, for patients with incurable cancer. We will show the possible survival benefit as well as the benefit of QOL of AIT, especially in biliary cancer patients treated with chemotherapy.

MATERIALS AND METHODS

Study design

This treatment is an observational cohort study conducted in Kawasaki Medical School Hospital between May 2009 and August 2012. All patient participants had a diagnosis of incurable cancer with a performance status capable of visiting our outpatient clinic and signed informed consent. Patients were excluded if they met the following criteria of consecutive use of steroid or immunosuppressant, autoimmune diseases, difficulty to manage at outpatient clinic, and uncontrolled complications. Participants were considered for study until deceased, withdrawal of consent, or follow-up contact was lost. There were no protocol-specified treatments or assessments. All aspects of patients' treatments over time, including specific chemotherapy agents and/or combinations, and the dose, schedule, and duration of AIT, were determined by a physician. This retrospective analysis was reviewed and approved by a central institutional review board (No. 1050).

ZAK cell generation and transfer

Heparinized venous blood (10 ml) was obtained from patients, and buffy coat and plasma were immediately separated by centrifugation (2,000 rpm, 30 min). The buffy coat was resuspended in RPMI-1640 medium, and the suspension was layered on Lymphoprep (Muto Pure Chemicals, Tokyo). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (2,000 rpm, 30 min) and washed twice. ZAK cell generation has been mentioned in detail elsewhere [10]. PBMCs were resuspended in the medium containing 2% heat-inactivated autologous plasma, 100 U/ml IL-2 (Sionogi, Osaka), and 1 μM zoledronate (Novartis, Tokyo) at a density of 1 x 10^7/ml. After incubation in a humidified atmosphere of 5% CO_2 for 24 hours at 37℃, cells (2 x 10^6/ml) were transferred into new medium containing plasma and IL-2 except of zoledronate (complete medium, CM), followed by further incubation for 10 to 14 days. CM was changed every 3 or 4 days. Cells were harvested by centrifugation, washed twice, resuspended in 100 ml saline after filtering through 200 μm mesh (Becton Dickinson), and administered intravenously for 30 min every 3 weeks. At each infusion, patients had blood drawn to prepare ZAK cells for the next transfer. Bacterial and endotoxin examinations were completed before each administration to make sure no contaminations.

Clinical efficacy

Survival of the patients was collected from patient record. If unknown, prognosis was requested by mail to the doctor-in-charge. Objective tumor response was evaluated by computed tomographic examinations. Data were collected at baseline and every 3 months. Complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) was determined by the investigator according to the RECIST [11].

QOL analysis

Assessment of quality of life was performed by FACT-BRM [12] before and after 3 administrations of ZAK cells. Documents were collected by research coordinators and analyzed independently of physicians.

Statistics

Statistical analysis was conducted on the SPSS program. Survival curve was drawn by Kapran-Meyer analysis to estimate median survival time (MST). For QOL analysis, paired Student's *t* -test was carried out. Values are presented as mean ± standard deviation, and $p<0.05$ was defined as statistically significant.

RESULTS

Characteristics of patients enrolled

Two hundred and twenty-eight patients including 134 males and 94 females, ranging 28 to 88 in age were entered in the trial, although 30 patients could not be treated with AIT because of disease progression in 26 patients and no lymphocyte growth in 4 patients (Table 1). Primary lesion of cancer consisted of 54 pancreatic, 43 colorectal, 31 stomach, 17 biliary, 14 lung, 10 esophageal, 10 breast, and 50 other organ cancers. Metastatic organs were liver, lung, lymph nodes, peritoneum, bone, and brain, and most patients were received concurrent anti-cancer treatment, as shown in Table 1.

Feasibility of ZAK cell generation and transfer

Generation of ZAK cells was carried out 1,359 times in total, and 1,277 cultures of those (94.0%) were uneventful (Table 2). Transfer of ZAK cells were completed once to 4 times for 90, 5 to 9 times for 75, 10 to 19 times for 20, 20 to 29 times for 9, and more than 30 times for 4 patients, a mean value of which was 6.8 times. A mean number of total cells transferred was 5.4×10^9 cells in all the patients treated, and 8.7×10^9 cells in those treated more than 5 times. No detection of bacteria and endotoxin was evidenced through all the cultures.

Table 1. Patients enrolled in the AIT trial

Total No.	228
Male/Female	134/94
Treated/untreated	198/30
Age (range, mean)	28-88, 60.7
Primary organ	
Pancreas	54
Colorectal	43
Stomach	31
Biliary	17
Lung	14
Esophagus	10
Breast	10
Others	50
Metastasis	
Liver	65
lung	55
Lymph node	32
Peritoneum	24
Bone	23
Brain	3
Concurrent treatments	
Chemotherapy	138
Radiation	3
None	59

Table 2. Feasibility of AIT

Total culture No.	1,359
Success of culture	1,277 (94.0%)
Administration No.	
0	30*
1-4	90
5-9	75
10-19	20
20-29	9
30<	4
(mean 6.8 times)	
Total cell No. administered (mean)	
All Pts treated	5.4 x 10^9
Pts treated 5 times<	8.7 x 10^9
Contamination detected	0 (1)**
Endotoxin >4.0	0

* 4, no lymphocyte growth; 26, disease progression
**Once positive, but finally negative at reexamination

Survival analysis

Survival analysis was summarized in Table 3. Under the median follow-up time of 8.9 months (range: 0.9-35.5), median survival time (MST) was 8.0 in lung, 5.4 in stomach, 14.6 in colorectal, 5.7 in pancreatic, and 11.9 months in biliary cancer patients.

Tumor response

Tumor response was shown in Table 4. Of 198 patients treated, 108 patients received more than 5 administrations of ZAK cells, of which 68 were evaluable for objective tumor responses. CR was observed in 3 including pancreas, biliary, and breast cancer patients, in whom oral fluorouracil was concomitantly administered with ZAK cells. PR was shown in 6 including 5 colorectal and one breast cancer patients. Response rate was 9 in 68 patients evaluated (13.2 %). No objective tumor response was evident in lung and stomach cancer patients. However, 40 patients (58.8 %) showed SD status, so that tumor control rate was estimated as 72.1 %.

Table 3. Overall survival

	MST (months)	95% CI (months)
Lung	8.0	6.1-10.0
Stomach	5.4	0.5-10.4
Colorectal	14.6	7.6-21.6
Pancreas	5.7	3.6-7.8
Biliary	11.9	5.1-18.8

Table 4. Objective responses treated with AIT

	Objective responses				
	CR	PR	SD	PD	Total
Lung	0	0	4	1	5
Stomach	0	0	10	2	12
Colorectal	0	5	9	5	19
Pancreatic	1	0	11	5	17
Biliary	1	0	5	3	9
Breast	1	1	3	6	9
Total	3	6	40	19	68

Adverse events

Of 198 patients treated, 2 showed temporally low grade fever after ZAK cell administration. No adverse events relevant with ZAK cell transfer higher than grade 2 were observed in all the patients treated.

QOL analysis

QOL analysis was shown in Table 5. The scores of social and emotional well-being were significantly improved after ZAK cell administration ($p<0.05$), as shown in Table 4. In the assessment of physical or functional well-being, some questionnaires showed trends to be improved after ZAK cell transfers ($p<0.1$). Moreover, the improvement of physical and mental queries was replied from 65 of 198 (33 %) patients at a regular hospital visit after 3 ZAK cell transfers.

DISCUSSION

We conducted an observation study of AIT using autologous lymphocytes activated *ex vivo* with antigen stimulation, including ZAK cells, in

Table 5. QOL analysis according to FACT-BRM

	before	**after**	**difference**	**p value**
PHYSICAL WELL-BEING				
I am bothered by side effects of treatment	1.10	0.81	-0.288	0.075
SOCIAL/FAMILY WELL-BEING	1.61	1.94	+0.327	0.077
I get support from my friends	3.31	3.61	+0.294	0.003
I feel close to my partner (or the person who is my main support)				
EMOTIONAL WELL-BEING	2.27	1.92	-0.346	0.038
I worry that my condition will get worse				
FUNCTIONAL WELL-BEING				
I am able to enjoy life	2.22	2.51	+0.294	0.062
I have accepted my illness	2.87	3.06	+0.192	0.096

treating patients with incurable cancer. Through more than 1,000 generations of ZAK cells, 94% cultures were uneventful without contaminations, indicating the safety and feasibility of our system for preparing ZAK cells. Our system can permit ZAK cell generation in a patient with almost no limitation. That more transfers of ZAK cells may be more effective for cancer treatments suggests the requirement of and need for a large-scale cell processing center in our hospital. In addition to this proposal, it is worth noting the difficulties when considering that the law of good manufacturing practice (GMP)/good tissue practice (GTP) for cell-engineering therapy has not yet been established in Japan, although it is highly accelerated, recently [13]. This law is urgently required and necessary for future progress in this field as well as in regenerative medicine.

The survival analysis implied marginal benefits in a few cancer types and a promising benefit in biliary tract cancer patients, whose OS was 11.9 months. Sasaki *et al*. conducted a randomized phase II study of gemcitabine and S-1 combination therapy versus gemcitabine monotherapy for advanced biliary tract cancer, and demonstrated that overall survivals of these two treatments were 8.9 vs. 9.2 months, respectively [14]. That the most patients in our observational study had been treated with chemotherapy before our ZAK cell treatment makes our 11.9 months of OS in biliary tract cancer patients highly significant. Shimizu *et al*. demonstrated that the 5-year progression-free survival (PFS) and overall survival (OS) were prolonged in the group receiving AIT plus vaccination with autologous tumor lysate-pulsed DCs compared with those receiving surgery alone [15], indicating a survival benefit of AIT in the biliary cancers. Moreover, in our pancreatic cancer cohort, patients who received ZAK cells more than 5 times showed MST of 12.1 months (data not shown). Kawaoka *et al*. reported that AIT using activated autologous lymphocytes stimulated by the MUC1-expressing human pancreatic cancer cell line prolonged the survival with reducing liver metastases after surgery [16], also indicating a possible survival benefit of AIT in pancreatic cancer. Now, we are conducting a new AIT trial of a phase II setting using ZAK cells combined with oral S-1 administration as the first line treatment for patients with unresectable or metastatic pancreatic cancer. Here, we have to search adequate biomarkers that predict patients suitable for ZAK cell transfers.

In contrast to the survival benefit, objective tumor response was evident in a few patients (13.2 %) treated in combination with chemotherapy. It was reported that objective tumor response was shown in only one of more than 300 prostatic cancer patients enrolled in a vaccine trial of the sipuleucel-T [17], suggesting that immunotherapy may prolong the survival without tumor shrinkage. Researchers in this field should pay more attention on this survival benefit of immunotherapy.

Most importantly, AIT with ZAK cell transfer showed the improvement of QOL in patients with incurable cancer. Wu *et al*. demonstrated that QOL assessments were significantly improved in lung cancer patients treated with chemotherapy plus cytokine-induced killer lymphocytes compared to those treated with chemotherapy alone [18], suggesting the QOL benefit of AIT. Although we have informed correctly the 'not-yet-proven' efficacy of our AIT for survival and tumor response benefit in this prospective study, 228 patients were rapidly enrolled during 3 years and 3 months, which is possibly indicative of the number of patients nationwide willing to seek alternative and additional treatments after experiencing the failure of standard therapies in Japan. Many patients continue to search for promising and successful cancer treatments, even those accepting their own fate of being terminally ill. For these patients, we have a responsibility and duty to proactively prepare adequate supportive care systems, like the ZAK cell transfer. It is highly

warranted that AIT using autologous activated lymphocyte, including ZAK cells, should be established urgently based on the views, not only for tumor eradication and prolonging patient survival, but importantly also for improving peoples quality of life.

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