Activated cytotoxic T-lymphocyte immunotherapy is effective for advanced oral and maxillofacial cancers

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Abstract. Conventional cancer treatments are surgery, radiotherapy, and chemotherapy, but treatment efficiency is insufficient and cancer recurrence is common. Immunotherapy has been added as an important cancer treatment component, but no reports on its efficacy in oral and maxillofacial cancers exist. We evaluated the clinical efficacy of adoptive immunotherapy using *ex vivo*-activated cytotoxic T lymphocytes (CTL) in the treatment of 7 patients with advanced oral and maxillofacial cancers with stage IV disease at diagnosis. The mean follow-up period was 26.2 months. Phenotype of the lymphocyte assay revealed that the percentage of CD4+ T cells decreased and that of CD8+ T cells increased among infused lymphocytes compared to that in unstimulated peripheral blood mononuclear cells (PBMCs), and infused lymphocytes produced a significantly higher level of IFN-γ than PBMCs or tumor cells alone. In a representative patient who refused surgery tumor regression was confirmed after CTL infusion. Computed tomography clearly indicated a significant reduction in tumor size followed by the complete disappearance of the tumor. Histological examination showed that the cancers in patients receiving CTL therapy were heavily infiltrated with lymphocytes. The other 2 patients who received CTL therapy as adjuvant therapy showed neither recurrent disease nor new disease lesions. The 1-year survival rates showing response and those with progressive disease were 100 and 25%, respectively. Moreover, no significant adverse reactions were reported during the study period. CTL therapy remains in the early stages of treatment options, but it has potential as a valuable treatment and improvement of quality of life for patients with otherwise incurable cancers.

Introduction

Cancer incurs significant morbidity and mortality, with >6 million deaths occurring each year worldwide (1). Until recently, cancer treatment was a 3-pronged approach, comprising surgery, radiation therapy, and chemotherapy (2). However, cancer treatment efficacy is frequently inadequate and disease is often recurrent, and in such cases, virtually all patients eventually succumb to the disease. Patients with recurrent or metastatic disease are generally incurable and are not eligible for multimodal curative treatment. The goals of cancer treatment are prolongation of overall survival, palliation of existing symptoms, prevention of new cancer-related symptoms, and improvement in the quality of life (3). Therefore, the development of new and effective therapeutic strategies of cancer is necessary.

In the past 25 years, immunotherapy has been added as an important component of cancer treatment and is expected to provide a new strategy for cancer therapy (2). Rosenberg *et al* (4) introduced lymphokine-activated killer cell (LAK) therapy as a form of immunotherapy, but frequent adverse reactions to those cells activated with IL-2 were found and an adequate clinical effect was not achieved, resulting in a decrease in favor for the LAK therapy (5,6). Recently, it has become widely accepted that cytotoxic T lymphocytes (CTL), originally isolated from tumor-infiltrating lymphocytes (TILs) *in vitro* (7), play a major role in tumor rejection *in vivo* (8). This approach involves stimulation of T lymphocytes with a specific tumor antigen *in vitro*. It is known that lymphocytes rapidly express and secrete cytokines after stimulation with appropriate antigens, and the quantity of IFN-γ produced indicates the reactivity of lymphocytes for tumor cells (9). The number of tumor-specific T cells may also be enhanced by repetitive stimulation with autologous tumor cells or antigen *in vitro*. The immune system recognizes tumors, but the tumor

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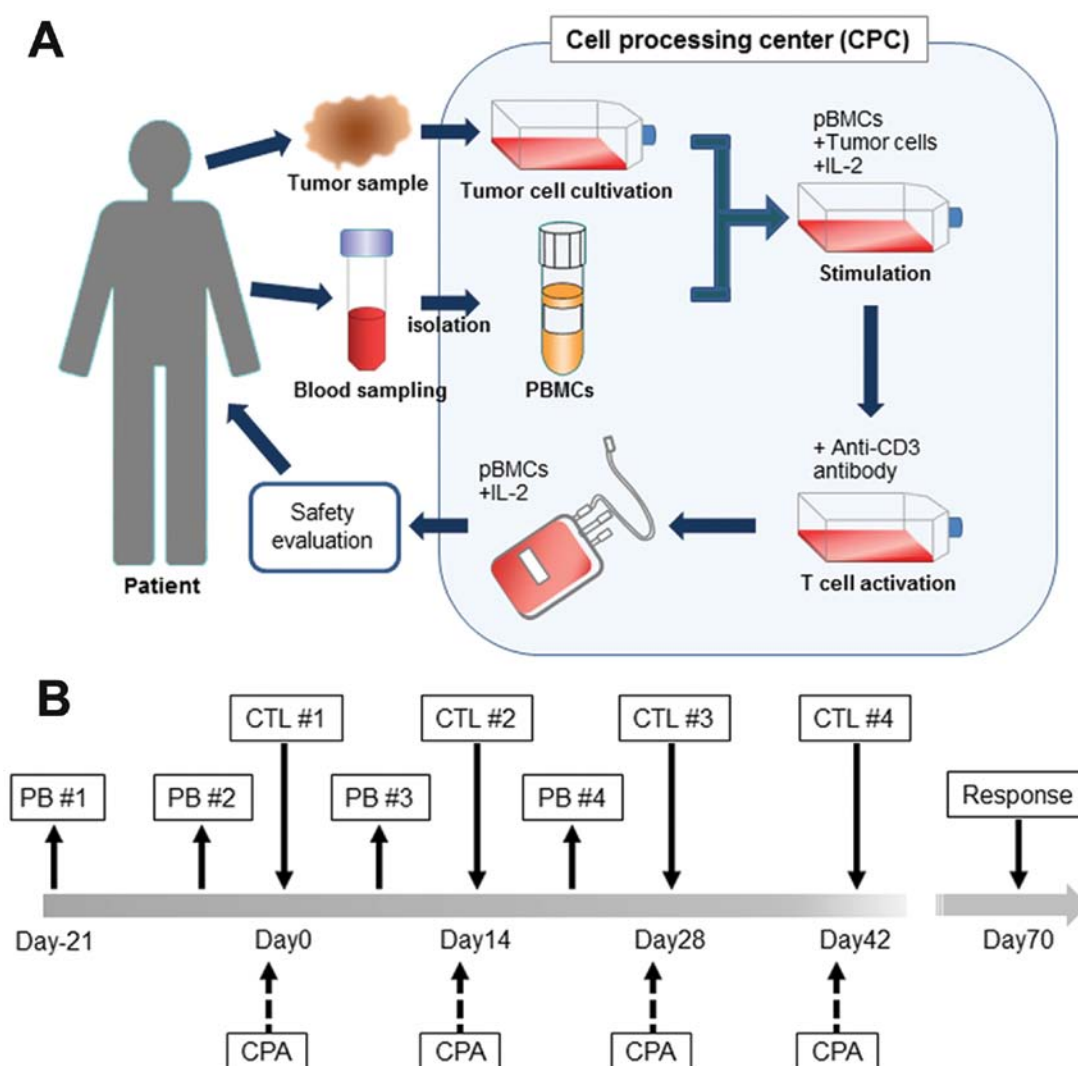E-mail: yyamada0925@gmail.com

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Figure 1. Treatment protocol of cytotoxic T lymphocyte (CTL) treatment. (A) The schema of CTL preparation. (B) Treatment schedule. PB, peripheral blood sampling; CPA, cyclophosphamide.

microenvironment generates immunosuppressive cells leading to immune evasion of cancer. The approach to overcoming the immunosuppressive tumor microenvironment is to generate cells *in vitro* that will kill tumor cells. Cellular immunotherapy, in the form of CTL, has been successfully used to treat virus-associated malignancies, some hematological malignancies, and some solid tumors (10-12). Antigen-specific CD8+ T cell clones have also been shown to be effective for treating malignant disease (13), implying T-cell specificity to tumor cell antigens.

Oropharyngeal cancer is the eighth most common cancer worldwide with over 145,500 deaths occurring every year (14). It is widely known that lymph node metastasis occurs at a high frequency because of the rich lymphatic submucosal plexus of the oropharynx, resulting in decreased survival rates. Although the conventional treatment for oral and maxillofacial cancers is surgery, radiotherapy, and chemotherapy, surgical treatment can often lead to severe morbidity and decreased quality of life. Chemotherapy is also widely used in cases of oral cancer, but the effects are limited because of lack of selectivity, narrow therapeutic margins, and the high prevalence of drug resistance (14). As a result, new therapeutic options for oral

and maxillofacial cancers are urgently required. However, no studies on the use of immunotherapy in oral and maxillofacial cancers have been reported to date.

In this study, we applied *ex vivo* stimulation of CTL using irradiated tumor cells, which were used in an attempt to augment host immunological response, to generate killer cells for adoptive immunotherapy. Then, we investigated the clinical effects of CTL therapy in advanced oral and maxillofacial cancers.

Materials and methods

Study entry criteria and patients. Patients were eligible for this study if they had oral and maxillofacial cancers; a clinical performance status of 0, 1, or 2; if their tumor could be obtained for the CTL treatment and was positive for HLA class I antibody. Patients were excluded from participation if they were positive for hepatitis B or C antigens or for HIV antibodies. Seven patients aged between 59 and 79 years (mean age, 68.4 years) were enrolled in this study. This study was approved by the Ethics Committee of Aichi Medical University and written informed consent was obtained from all the patients.

Table I. Patient characteristics and clinical summary.

Patient no.	Age (years)	Sex	Disease site	Diagnosis (stage)	Previous treatments	Total no. of injected cells x10^9 (total no. of injections)	Cyclophosphamide (mg/mm^2)	Observation periods (months)
1	69	M	Floor of the oral cavity	SCC (IV)	RT, chemo	29.4 (20)	129	46
2	79	M	Mandible gingiva	Malignant melanoma (IV)	Surgery	7.16 (4)	129	60
3	66	M	Maxillary gingiva	SCC (IV)	RT, chemo, surgery	4.12 (4)	119.8	0.5
4	65	M	Oropharynx, buccal mucosa, maxillary gingiva	SCC (IV)	RT, chemo, surgery	6.4 (4)	127.4	3
5	59	F	Tongue	SCC (IV)	RT, chemo, surgery	6.5 (4)	135.1	58
6	72	M	Tongue	SCC (IV)	Surgery	5.38 (4)	126.6	14
7	69	F	Maxillary gingiva	Spindle cell sarcoma (IV)	Chemo, surgery	10 (4)	155	2

SCC, squamous cell carcinoma; RT, radiotherapy; chemo, chemotherapy.

Preparation of cells. Tumor samples used for the stimulation of peripheral blood mononuclear cells (PBMCs) were obtained from each patient and incubated for 1 h in RPMI-1640 (Nipro, Japan) containing 1,250 U/ml penicillin, 1,250 µg/ml streptomycin and 31.25 µg/ml amphotericin B, after which they were minced and enzymatically digested with 2% collagenase for 1 h at 37°C in 5% CO_2 in an incubator. The tumor cells were cryopreserved at -152°C until they were used for stimulation.

Peripheral blood samples (40 ml) were collected from the patients and PBMCs were obtained by the gradient method using Lymphosepar I (Immuno-Biological Laboratories Co., Ltd., Fujioka, Gunma, Japan). PBMCs were incubated with TIL-Medium I (Immuno-Biological Laboratories Co., Ltd.) containing 2.5% autologous plasma, 50 U/ml IL-2 (Shionogi & Co., Ltd., Osaka, Japan), and tumor cells irradiated for 4-5 days (Fig. 1A). PBMCs stimulated with tumor cells and IL-2 were then cultured in an anti-CD3 antibody (Janssen Pharmaceutical, Tokyo, Japan)-coated flask for 4-6 days to activate the T cells. At the end of this period, PBMCs were transferred into a culture bag (GT-T610 culture bag, KB0001, Takara Bio Inc., Otsu, Shiga, Japan) and cultured with GT-T503 culture medium (KB503S, Takara Bio Inc.) containing 2.5% autologous plasma and 50 U/ml IL-2 for 10-12 days before use for CTL therapy. The safety of the PBMCs was examined using the BD BACTEC™ System (Becton-Dickinson and Company, Tokyo, Japan), MycoAlert™ (Eidia, Ibaraki, Japan), and Endosafe-PTS (Wako, Japan).

Treatment schedule. Four infusions of CTL, scheduled at 2-week intervals, constituted 1 cycle and were administered intravenously (Fig. 1B). Before CTL infusion, all patients received cyclophosphamide at a dose of 200 mg/body (Shionogii & Co., Ltd) to inhibit the activity of regulatory T cells. Patient response was assessed using computed tomography (CT) and physical examination, on the basis of Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.

Phenotype assays. Phenotype analysis of infused lymphocytes as CTL was analyzed by flow cytometry analysis with a cytomics FC500 (Beckman Coulter, Inc., Brea, CA, USA). Cultured cells were stained with anti-CD2/anti-CD20 (T11-RD1/B1-FITC, 6603928, Beckman Coulter, Inc.), anti-CD4/anti-CD8 (T4-RD1/T8-FITC, 6603802, Beckman Coulter, Inc.) and anti-CD56 (CD56-PE, N901, A07788, Beckman Coulter, Inc.) according to the manufacturer's instructions.

IFN-γ released from CTL in response to autologous tumor cells was analyzed according to a previously reported method (15). Briefly, CTL were incubated with tumor cells overnight at 37°C in 5% CO_2. The supernatants were collected and the amount of IFN-γ was measured using an ELISA kit (Human IFN-γ ELISA Ready-Set-Go, 88-7316-88, eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Results

Patient characteristics. A total of 44 T-cell infusions were administered to 7 patients (5 males and 2 females) diagnosed with oral and maxillofacial cancers (Table I). Five patients had squamous cell carcinoma (SCC), 1 patient had malignant melanoma, and the remaining patient had spindle cell sarcoma. All
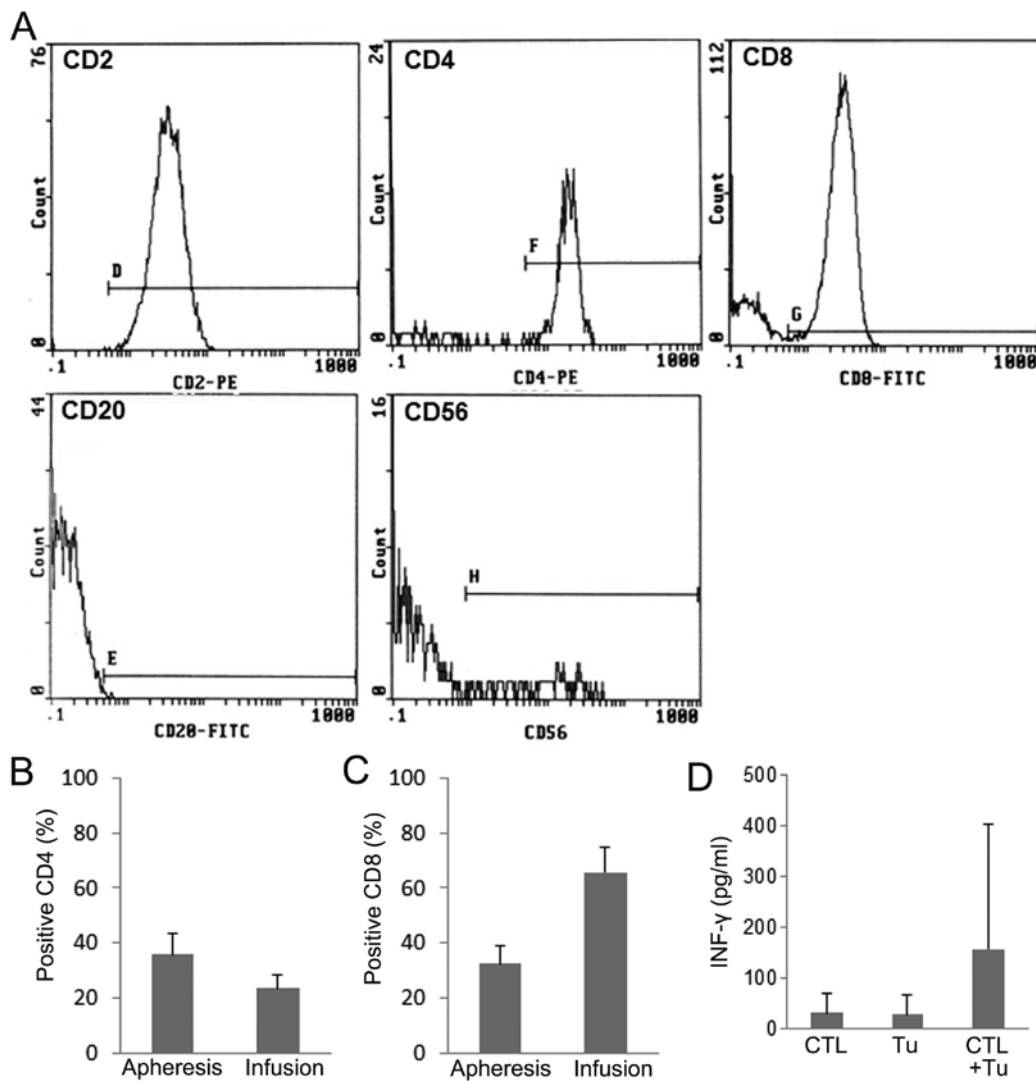
Figure 2. Characteristics of cytotoxic T lymphocytes (CTL). (A) Results of typical flow cytometric analysis for expression of surface markers (CD2, CD4, CD8, CD20 and CD56) by CTL before infusion. (B and C) Changes in surface marker expression of helper T cells (CD4) and cytotoxic T cells (CD8) before stimulation (apheresis) and after stimulation (infusion). Data shown in the bar graph are the means ± standard deviation. (D) CTL activity, assessed by production of IFN-γ was measured. Tu, tumor cells.

patients presented with stage IV disease at diagnosis. Tumor cells from these patients expressed HLA class I antigen by the means of immunostaining, indicating that tumor antigens could be recognized by CTL (data not shown). All patients had received prior treatment, including chemotherapy, radiotherapy, or surgery. In 5 cases (cases 1, 3, 4, 6 and 7), because the patients did not respond to previous conventional therapy, they were switched to CTL therapy. Two patients (cases 2 and 5) received CTL therapy as adjuvant therapy.

Characteristics and activity of CTL. To characterize CTL, surface markers were analyzed. CTL were mostly positive for CD2 (99.3±0.3%), and partially positive for CD56 (10.9±11.1%) (Fig. 2A). The expression of CD4, the marker of helper T cells in CTL, decreased to 23.6±4.5%, whereas that in peripheral blood mononuclear cells (PBMCs) before stimulation was considerably higher (35.7±9.1%) (Fig. 2B). In contrast, the expression of CD8, the marker of cytotoxic T cells, in CTL increased from 32.5±6.5 to 65.6±9.3% after stimulation (Fig. 2C). The activity of PBMCs stimulated with autologous

tumor cells and IL-2 (CTL) was assessed by measuring IFN-γ production (Fig. 2D). CTL produced a significantly higher level of IFN-γ than unstimulated PBMCs or tumor cells alone (Tu).

Clinical results. The total number of administered cells per patient ranged from 4.12-29.4x10^9 cells. The mean dose of cyclophosphamide received was 131.7 mg/mm^2 body surface area (range, 119.8-155 mg/mm^2 body surface area). The mean observation period was 26.2 months (range, 0.5-60 months).

The representative patient (case 1 in Table I) attaining CR had SCC of the floor of the oral cavity (T4N2cM0, stage IV) with lymph node metastases at diagnosis. The patient had been treated with radiotherapy and chemotherapy, but the disease was refractory. The patient refused surgery, but chose CTL treatment because of the high degree of invasiveness and the treatment burden necessitating resection as far as the skin, including resection of most of the mandible (Fig. 3A, B, E and F). First, the lymph node metastatic lesions were resected before CTL treatment, after which CTL infu-
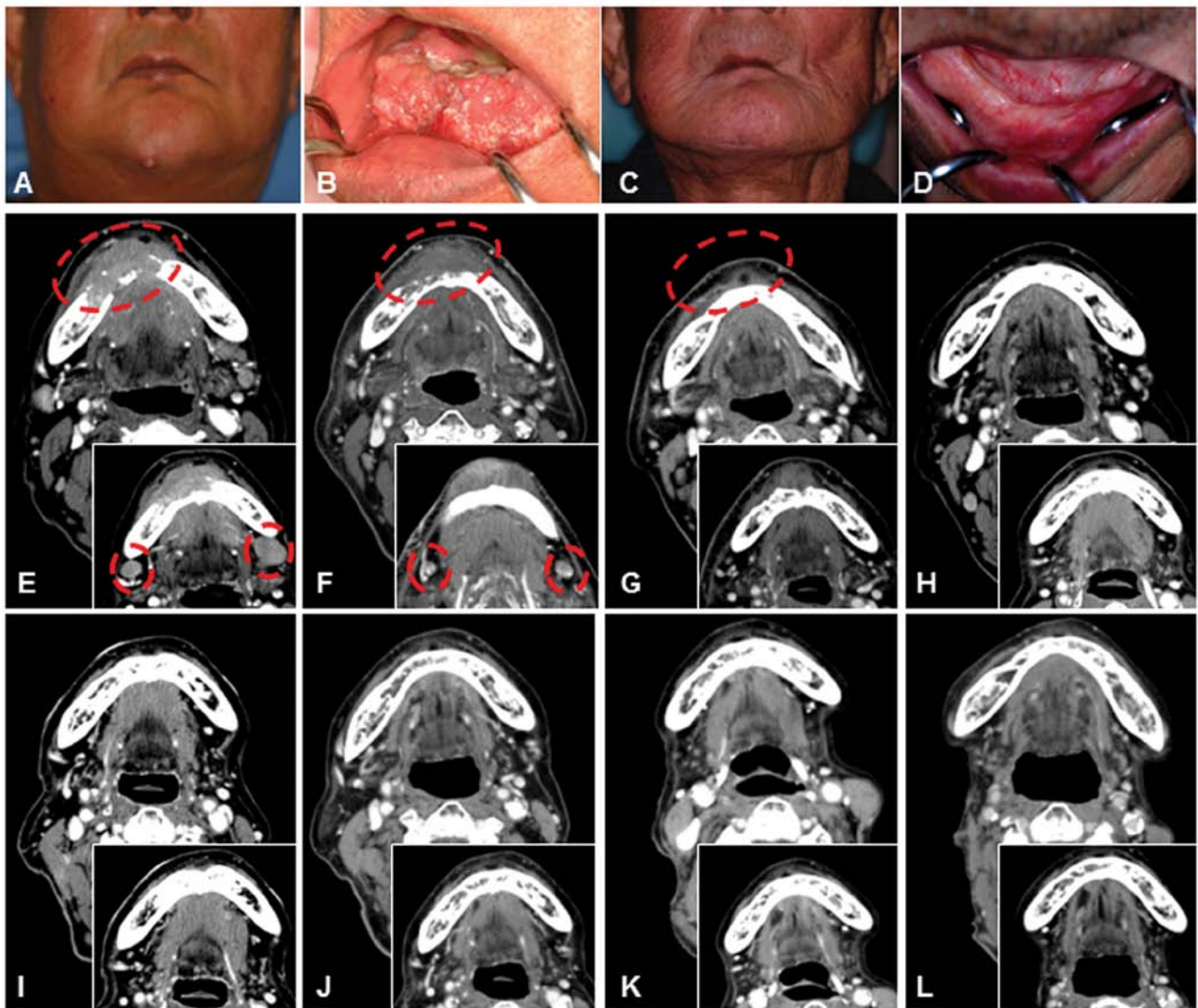
Figure 3. Clinical outcomes in a representative patient (case 1). (A and B) Extraoral and intraoral findings of the patient before treatment. (C and D) Extraoral and intraoral findings 44 months later, after 5 cycles of treatment. (E-L) Computed tomograpy image taken at diagnosis (E), after radiotherapy and chemotherapy (F), after 2 cycles (G), 4 cycles (H), and 5 cycles of cytotoxic T lymphocyte treatment (I), 10 months (J), 36 months (K), and 46 months (L) after the final cycle of treatment.
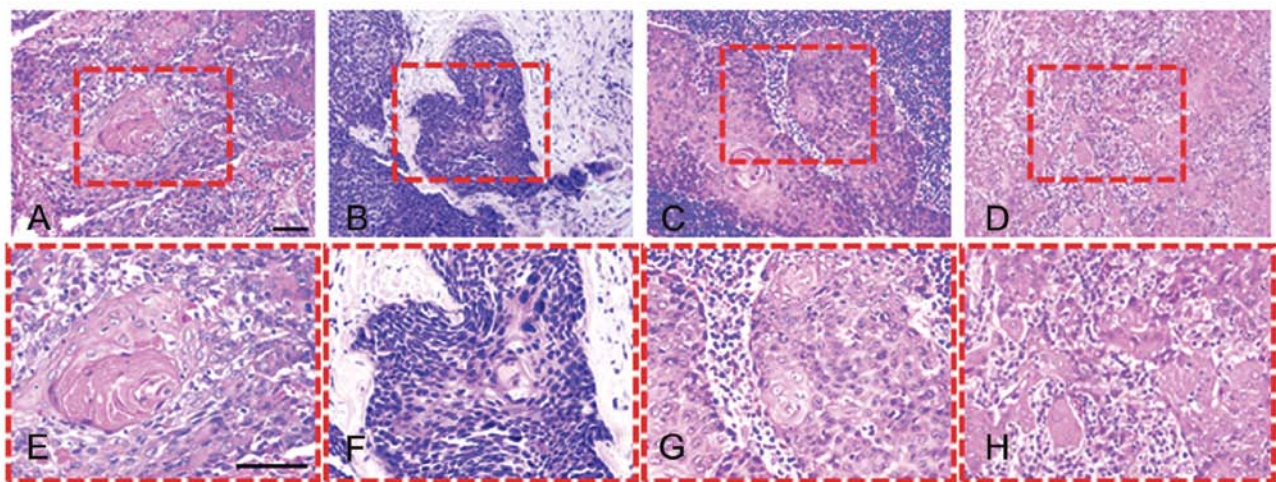

Figure 4. Representative histological images of biopsy samples. (A-D) Biopsy samples were analyzed by hematoxylin and eosin staining. (E-H) Higher magnification images of (A-D). (A and E) Well-differentiated squamous cell carcinoma cells were seen before treatment. (B and F) After chemoradiotherapy, residual cancer cells and irregular chromatin was observed. (C and G) Lymph node biopsy prior to the 1st infusion. (D and H) After 4 cycles of cytotoxic T lymphocyte treatment, tumors were infiltrated with lymphocytes. Scale bar, 50 μm.
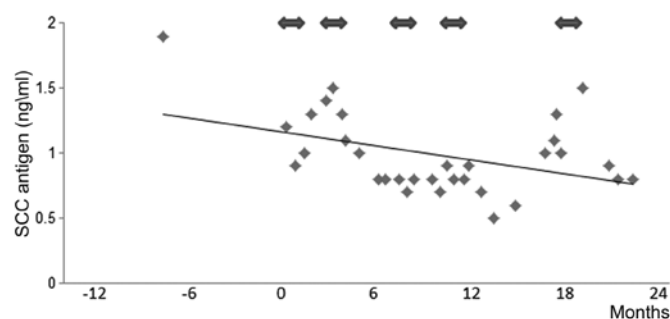
Figure 5. Changes in the serum SCC antigen. The arrows indicate the times of cytotoxic T lymphocyte treatments.
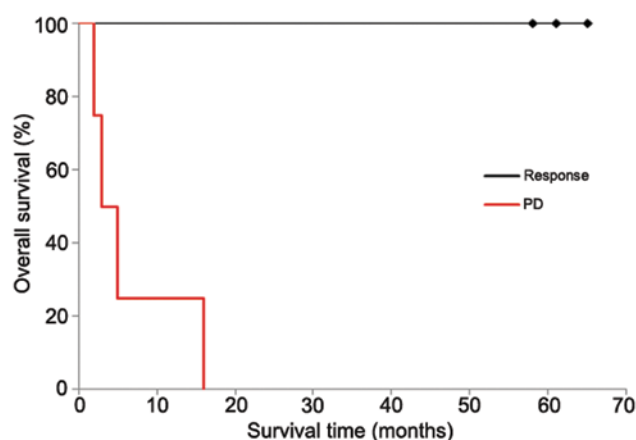

Figure 6. Patient survival data. Kaplan-Meier plot showing the duration of survival of patients treated with cytotoxic T lymphocytes. Dots indicate patients who are still alive and being followed up in the study. Response, response group, PD, progressive disease group.

sion was performed by IVH injection after culture. After the second course of CTL infusion, the tumor size was markedly decreased (Fig. 3G). Because of the partial response, this patient received additional courses of CTL therapy at 7, 11 and 18 months after the first course of CTL infusion. Computed tomography (CT) clearly indicated a significant reduction in tumor size followed by the complete disappearance of the tumor (Fig. 3H and I). Target lesions remained in stable CR for 46 months after the last course of CTL infusion (Fig. 3C, D and J-L). Histological examination of clinical biopsy samples showed well-differentiated SCC cells with individual cell keratinization and formation of cancer pearls before treatment (Fig. 4A and E). After chemoradiotherapy, residual cancer showed a wide pattern of alterations and the nuclei of residual neoplastic cells were enlarged and irregular, with clumped chromatin (Fig. 4B and F). The lymph node exenterated for the 1st cycle of CTL therapy revealed the presence of cancer cell nests (Fig. 4C and G). Tumors treated with CTL were heavily infiltrated with lymphocytes (Fig. 4D and H). In addition, the SCC antigen levels decreased after CTL treatment (Fig. 5).

Two patients (cases 2 and 5) who received CTL therapy as adjuvant therapy showed neither recurrent disease nor new disease lesions during the observation period. In cases 6 and 7, the target tumor lesions resulted in stable disease (SD) after CTL treatment. The 1-year survival rates of the response group (cases 1, 2 and 5) and the progressive disease (PD) group (cases 3, 4, 6 and 7) were 100 and 25%, respectively (Fig. 6). Moreover, no significant adverse reactions were reported during the observation period.

Discussion

This study evaluated the clinical efficacy of adoptive immunotherapy using specific lymphocytes activated by irradiated autologous tumor cells, which expressed HLA class I antigen, and used as immunogens to patient PBMCs for the treatment of advanced oral and maxillofacial cancers. Our results with CTL immunotherapy in 7 patients with advanced cancers showed that adoptive cellular immunotherapy can be performed safely in such patients. Although the cells are expected to be utilized in both adoptive and active immunotherapies against cancer, the clinical efficacy of immunotherapy remains to be clarified. In a previous report, there was a tendency for CTL not to be activated from patients with low CD8+ cell populations (16). However, in this study the proportion of CD8+ cells in the CTL population was increased compared to that in the PBMCs before stimulation (Fig. 2C). Mature differentiated CD8+ T cells and some types of CD4+ T cells release IFN-γ, which enhances the immune response by upregulating the expression of MHC class I molecules on both tumor cells and tumor-resident APCs (17). Because the percentage of CD8+ cells increased after stimulation, higher IFN-γ production by CTL may have occurred in the patients included in this study. The successful induction of effective CTL from PBMCs may result in favorable clinical results.

Many clinical studies of adoptive immunotherapy using mostly LAK cells and TILs have shown a significant response rate in patients with melanoma and some other cancers (17,18). These studies also demonstrate efficacy prior to surgery (19). However, there has been no report of a clinical study investigating CTL administration without surgery and demonstrating obvious tumor regression of advanced oral or maxillofacial cancers. To our knowledge, our report is the first to show clear evidence for the potential of the application of immunotherapy in oral and maxillofacial cancer patients. In our study, the representative patient refused surgery and instead was treated with CTL immunotherapy. Interestingly, the original tumor and lymph node metastases disappeared following CTL treatment despite the absence of surgery (Fig. 3). Histological examination showed that the efficacy of CTL treatment, as evidenced by the fact that tumors were heavily infiltrated and attacked by the activated lymphocytes (Fig. 4). In addition, a positive response to CTL led to an improvement in overall survival for patients with advanced cancer. Moreover, no significant adverse reactions were reported during the observation period. Therefore, CTL induction using autologous tumor cells as immunogens has potential anticancer efficacy in refractory oral and maxillofacial cancers.

In agreement with our findings, Khammari *et al* (20) reported that adoptive immunotherapy with tumor-infiltrating lymphocytes used as an adjuvant regimen for stage III melanoma was effective and both relapse-free survival and overall survival were extended. Another study revealed an apparent survival benefit for patients with resected lung cancer who received adjuvant CTL (21). It has been reported that the rate

of local and regional recurrence of oral cancer varies from 18 to 76% according to the TNM stage and initial treatment, and recurrence has been reported in >50% patients with advanced clinical stages (III or IV) (22). In this study, patients with stage IV at diagnosis who received CTL treatment as adjuvant therapy, continue to have a good prognosis without recurrent disease (cases 2 and 5 in Table I). These results indicate that this treatment strategy might be effective as adjuvant treatment for patients with advanced stage cancer.

In conclusion, adoptive immunotherapy using *ex vivo* activated CTL with irradiated autologous tumor cells has favorable clinical efficacy without side effects. Therefore, it could form a novel treatment option that lead to improvement of quality of life for advanced oral and maxillofacial cancers and may be a promising approach for targeted therapy in such patients. However, further clinical trials will be required to confirm the present findings in a large-scale prospective clinical study.

Acknowledgements

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