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Establishment of interleukin 2 dependent cytotoxic T lymphocyte cell line specific for autologous brain tumor and its intracranial administration for therapy of the tumor

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Abstract

Autologous brain tumor specific CTLs were induced from the patient's PBL by a mixed lymphocyte-tumor culture, and were maintained for more than 2 months in a medium containing exogenous IL-2. The autologous T cell line containing specific CTL was administered into the tumor-bed for the treatment of malignant glioma. In 2 cases out of 5, tumors regressed more than 50% in diameter. One of these patients is still alive now with full of his social activities, and it is 104 weeks after the initiation of the immunotherapy. Autologous T cell lines were safely administered in all cases without any complications nor toxicities.

Introduction

There are accumulating reports that experimental tumors can be cured by injecting tumor-specific T lymphocytes which can be maintained in a medium containing IL-2 [1–5]. However, clinical trials with such a procedure on human neoplasms have been limited as yet. Since it is difficult to get the immune T cells specific for tumor in human, nonspecifically activated lymphocytes were administered for the therapy for human cancer; lymphokine activated killer (LAK) cells [6, 7, 9, 11], phytohemagglutinin-activated killer (PAK) cells [7, 8], or allo-activated killer (AAK) cells [10]. On the other hand, brain tumors were reported to be immunogenic [12–15], and in patients of brain tumors, brain tumor-specific killer cells were reported to be present in the peripheral blood lymphocytes (PBL) [16, 17]. Young tried to treat malignant glioma patients with autologous PBL stimulated with own tumor cells, and reported that such an immunotherapy resulted in the elongation of survival period [18, 19]. Therefore, we attempted in this trial to establish a T cell line having a specificity against autologous brain tumor, and to propagate the T cell line in an IL-2 containing medium for the adoptive immunotherapy for malignant glioma patients.

Materials and methods

Establishment of autologous T cell line containing specific cytotoxic lymphocytes (CTL) for own brain tumor

Peripheral blood lymphocytes (PBL) were re-

sensitized with autologous brain tumor cells in vitro. In brief, $0.5 - 1 \times 10^7$ PBL were mixed with 5×10^5 autologous cultured glioma cells, that was treated with Mitomycin C (MMC: 0.1 mg/ml) at 37 °C for 45 min, in 2 ml of a complete medium without IL-2 in a 24 well type tissue culture plate (Corning 25820). A complete medium represents an RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M of 2ME, 2 mM of L-glutamine, 1 mM of sodium pyruvate, and antibiotics. The cell mixture was incubated for 5 to 7 days in a CO_2 incubator. After the primary culture, survived PBLs were harvested, were washed once, and were resuspended in 5 ml of a complete medium containing exogenous IL-2 and were maintained in a culture flask (Corning 25100). As a source of exogenous IL-2, commercially available PHA-conditioned medium (IBL Inc., Gunma, Japan) or human recombinant IL-2 (rIL-2), supplied from Shionogi Co., Osaka, Japan, were used at the final concentrations of 10% or 100 U/ml, respectively.

Establishment of glioma cell line

Fresh brain tumor, obtained at a surgical operation, was minced by scissors into small fragments of less than 1 mm^3 and was suspended in 5 ml of a complete medium in a tissue culture flask. After 1 or 2 weeks, it became a monolayered glioma cell line. Glioma cells were harvested by 0.4% trypsin in a Hanks solution at 37 °C for 15 min, were washed 3 times, and were used for the stimulator of mixed lymphocyte-tumor culture or for the target cells in a CTL assay.

CTL assay

Conventional ^{51}Cr release assay was performed by mixing 1×10^4 ^{51}Cr-labelled-tumor cells with effector T cells at various effector-target (E/T) ratios in a 96 well-typed round-bottomed culture plate. Assay time was 4 hours for K 562 and 14 hours for brain tumors. Percent specific ^{51}Cr release was determined by a following formula.

$$\% \text{ specific } ^{51}Cr \text{ release} = \frac{X - B}{A - B} \times 100$$

where A is a maximum ^{51}Cr release from the target cells by 0.1% sodium deoxy cholate, B is a spontaneous ^{51}Cr release, and X is an experimental value.

Characterization of cell surface phenotype

Cells were stained with FITC-labelled monoclonal antibodies and were analysed by a cell sorter (FACS IV or FACStar, Becton Dickenson).

Panning method for the depletion of Leu 2^+ cells

In order to eliminate Leu 2^+ cells from an autologous T cell line containing specific CTL, 1×10^7 T cells were first reacted with 0.2 ml of 1 : 10 diluted anti-Leu 2 antibody, which had been dialysed against PBS to eliminate NaN_3, for 30 min on ice. Cells were washed once, were resuspended in 6 ml of 2% FCS-Hanks solution, and were poured into a 10 cm-plastic petri dish which was coated with purified goat antibodies against mouse immunoglobulins (goat anti-MIgs). Cells were panned on the dish at 4 °C for 1 hour with occasional swirlings as described [20]. Non-adherent cells, i.e., Leu 2^- cells, were gently recovered from the dish for further experiment.

Results and discussion

Establishment of autologous brain tumor-specific T cell line

It was reported that PBL of glioma patients showed the preferential cytolytic activity against autologous brain tumor cells [16, 17]. Although, it was not clear that the observed cytolysis was mediated by conventional CTL. We tried to establish a T cell line from PBL of a malignant glioma patient by re-sensitizing PBL with autologous brain tumor

cells (mixed lymphocyte-tumor culture) and subsequently to propagate the stimulated cells by exogenous IL-2.

Table 1 shows that the established T cell line from patient KIM killed preferentially the autologous brain tumor. Glioma cells from unrelated patients, SUG or WAT, were poorly lysed by the T cell line of KIM. However, this was not because these glioma cells being resistant to the cytolytic machinery, since these glioma cells were lysed by LAK cells that were induced from patient KIM's PBL. Actually, glioma cells of patient SUG were more susceptible for LAK cells than autologous glioma cells (KIM) (Table 1). Therefore, it was suspected that the preferential killing of autologous brain tumors might be mediated by conventional CTLs but not by NK cells nor LAK cells.

However, it was revealed that an established T cell line was composed from different lymphocyte-subsets (Table 2). Major phenotype was Leu 3a that is a phenotype of helper/inducer T cells. Only a small percentage (3.0–7.5%) of the cells expressed Leu 2a that is a phenotype of CTL as well as of LAK-effector cells. There were also Leu 7 or Leu 11 positive cells known as NK cells, which was consistent with the result that the T cell lines showed cytotoxic activities against NK sensitive tumor cells, K 562 (data not shown). In order to clarify that Leu 2 positive cells were effector cells of specific cytolysis, T cell line of patient UCH was depleted of Leu 2^+ cells by a panning method as described in Materials and methods. As shown in Table 3, Leu 2 depleted UCH cell line lost the cytolytic activity against autologous brain tumors, while sham-treated UCH cell line killed the specific target. Therefore, it was concluded that the effector cells were Leu 2^+ cells.

Since most of the cells in the T cell line belonged to a helper/inducer T cell subset, it was asked whether the T cell line produced IL-2 upon the stimulation with autologous tumor cells in the presence of MMC-treated PBL as a source of antigen presenting cells. However, KIM cell line did not produce a detectable amount of IL-2 measured by a CTLL assay (data not shown).

Table 1. Specific cytolysis of a T cell line derived from autologous mixed lymphocyte-tumor culture.

Effector cell	E/T ratio	% specific ^{51}Cr release from target glioma cells[c]		
		KIM	SUG	WAT
T cell line (KIM)[a]	10	37	14	6
	20	54	21	11
	40	65	26	13
	80	74	27	13
LAK cells from KIM[b]	20	16	38	25
	40	33	59	29

[a] A T cell line was established from the autologous mixed lymphocyte-brain tumor culture of glioma patient KIM, and was maintained in an IL-2 containing medium.

[b] LAK cells were induced from PBL of patient KIM by culturing PBL in a complete medium containing 100 U/ml of rIL-2 for 3 days.

[c] Glioma cell lines were established from patients KIM, SUG and WAT, respectively.

Table 2. Cell surface phenotypes of T cell lines.

mc Ab[a] for	% of stained cells by mc Ab T cell line of patient		
	KIM	UCH	case 5
Leu 2a	3.0	3.2	7.5
Leu 3a	92.9	90.2	88.3
Leu 4	97.6	ND[b]	ND
Leu 7a	5.7	ND	1.7
Leu 11a	0.7	3.0	ND

[a] mc Ab represents monoclonal antibody.

[b] ND represents not done.

Table 3. Killer-effector cells for the autologous glioma cell-lysis were Leu 2 positive cells.

UCH cell line treated with[a]	% specific ^{51}Cr release[b]
none – anti-MIgs plate	28
anti-Leu 2 – anti-MIgs plate	0

[a] A T cell line of patient UCH was treated with anti-Leu 2 antibody, and was panned on an anti-MIgs coated-plate for the depletion of Leu 2^+ cells. As a control, sham-treated cells were panned on an anti-MIgs coated-plate.

[b] Glioma cells of patient UCH were ^{51}Cr-labelled and were used for target cells.

It is of interest that glioma cells are so unique to induce autologous CTLs in vitro. One possibility is that glioma cells are quite immunogenic compared with other tumors, since glioma cells are reported to possess tumor-specific or tumor-associated antigens such as onco-fetal antigens [12–15]. Another possibility is that the immunological status of a malignant glioma patient is not suppressed as are the cases in cancer patients of other neoplasms. Thus, in a glioma patient, tumor-reactive T cells are well preserved until the late stage of the disorder.

Administration of autologous T cell line specific for own glioma cells for the therapy of malignant glioma

Autologous CTL line specific for his own tumor was established from a malignant glioma patient and was administered into tumor-bed for the therapy of malignant glioma. Five patients were subjected to the trial (Table 4). Four out of them were glioblastoma multiforme (GM) patients and one was a malignant astrocytoma-oligodendroglioma (mixed glioma) patient. Two GM patients, cases 1 and 3, were recurrent cases and other 3 were primary cases. All five patients were operated surgically to remove or to probe tumors. At the surgical operation, a silicon tube was left to connect the tumor-bed with an Ommaya reservoir placed in the subgaleal space.

T cell lines and glioma cell lines were established from individual patients. Then, established T cell line was assessed for its specificity against autologous tumor cells. As shown in Table 5, each T cell line killed autologous glioma cells better than allogeneic glioma cells. In case 4, however, the T cell line killed the allogeneic tumor of case 3 as well as autologous glioma cells. On the other hand, the T cell line of case 3 killed autologous glioma cells well, but poorly cross-reacted with case 4's glioma cells. Such a one way cross-reactivity

Table 5. Preferential killing of autologous brain tumor cells by the individual T cell line[a].

Target cells	% specific ^{51}Cr release effector cells				
	case 1	case 2	case 3	case 4	case 5
case 1 GM[b]	70	ND[d]	ND	ND	ND
case 2 GM	ND	50	20	15	ND
case 3 GM	ND	20	70	60	ND
case 4 GM	ND	20	20	65	30
case 5 GM[c]	ND	ND	ND	ND	65
astrocytoma	20	10	20	20	ND
medulloblastoma	ND	ND	ND	ND	20
neuroblastoma	10	10	15	15	ND

[a] Cytotoxic activity of individual T cell line was assessed for the various brain tumor cell lines including autologous brain tumors at E/T ratio of 40.
[b] GM represents glioblastoma multiforme.
[c] MG represents mixed glioma.
[d] ND represents not done.

Table 4. List of patients subjected to the adoptive immunotherapy.

Case	Age (years)	Sex	Tumor	Operation	Other therapies before immunotherapy
1.	46	male	GM[a] (reccurence) left temporoparietal	partial removal 3 times	radiation, 6000 rad
2.	14	male	GM right thalamic	biopsy	none
3.	35	male	GM (reccurence) left temporoparietal	partial removal 2 times	radiation, 6000 rad ACNU 750 mg PSK 3.0 g/day
4.	73	male	GM left occipitoparietal	partial removal	none
5.	37	male	malignant mixed glioma[b], left frontal	partial removal	none

[a] GM is glioblastoma multiforme.
[b] Malignant astrocytoma-oligodendroglioma.

further supports the idea that the effector cells are conventional CTLs but not NK nor LAK cells [21]. Although, it was not determined yet that two glioma cell lines of case 3 and case 4 shared antigenic determinants, especially HLA antigens for the restriction element of killer T cells.

Specific cytotoxic activity was maintained in an IL-2 containing medium up to 13 weeks (Fig. 1). There were no degeneracy of the specificities happened during the course of the culture. It was noteworthy that one way cross-reactivity was always observed in case 4 (Fig. 1b).

In all cases, the T cell line specific for autologous brain tumor was dependent on exogenous IL-2 for its cell growth.

After informed consents were obtained from a patient and his family, autologous T cell line was administered into tumor-bed via an Ommaya reservoir. In principle, 5×10^7 cells in 2 ml of a Hanks solution were injected twice per week up to 13 times (Table 6). Therapeutic effects of this treatment were estimated by serial CT scannings and by per-

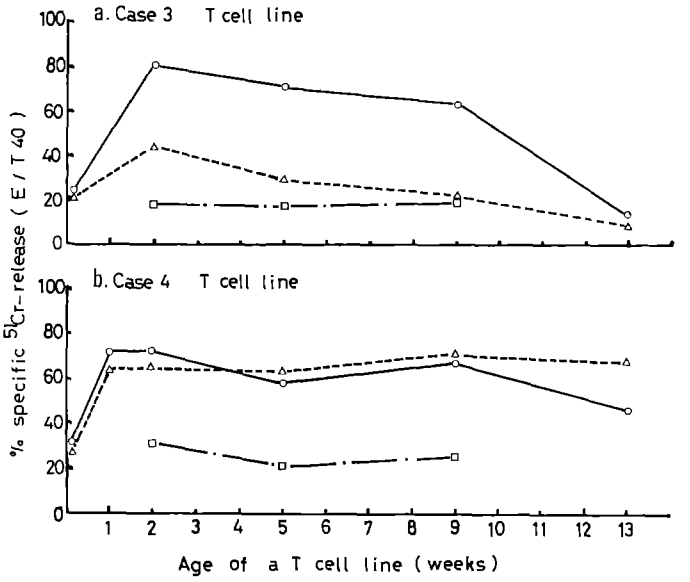

Fig. 1. Specific cytolytic activity was maintained up to 13 weeks of the culture. Cytotoxic activities of case 3 T cell line (Fig. 1a) or of case 4 T cell line (Fig. 1b) were monitored against autologous brain tumors, GM cell line of case 3 (o — — o) or GM cell line of case 4 (△ ----- △), respectively, as well as a third party allogeneic neuroblastoma (□ —·— □). Percent specific ^{51}Cr release was determined at E/T ratio of 40.

Table 6. Summary table of the adoptive immunotherapy using autologous T cell line.

case	adoptive immunotherapy			performance status		response (evaluated by CT)	side effects	survival time (weeks after 1st injection)
	number of injections	total cell number ($\times 10^8$)	duration of therapy (weeks)	pre-treatment	post-treatment			
1	10	7.2	10	50–60	30	PG	none	12 (died)
2	13	10.4	10	80	90	PR	none	52 (died)
3	8	5.6	6	70	20–30	PG	none	8 (died)
4	10	6.0	6	50–60	50	PG	none	18 (died)
5	7	5.0	4	90	100	PR	none	104 (alive)

PR: partial regression and PG: progression.

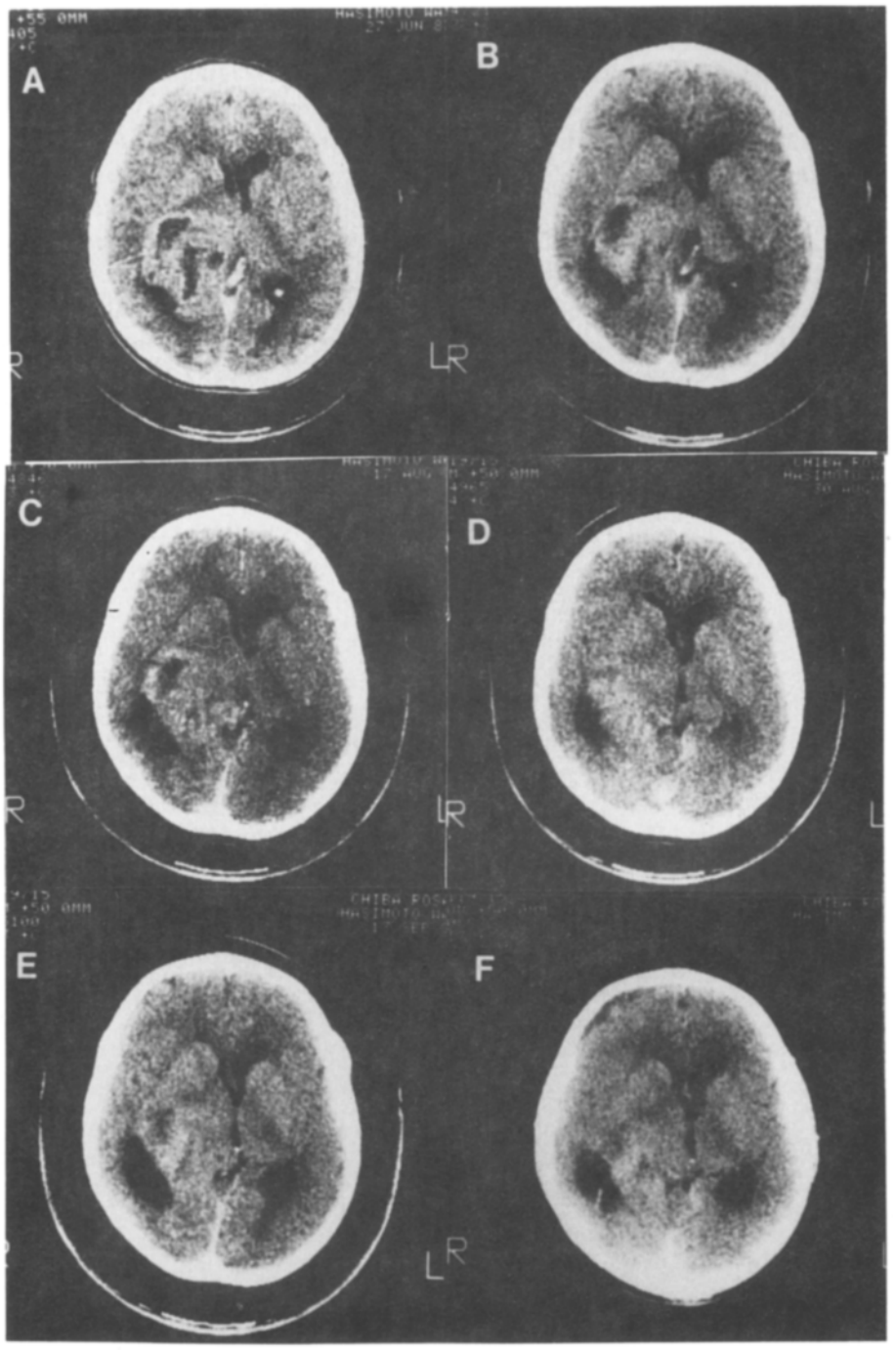

Fig. 2. Regression of the right thalamic glioblastoma multiforme of case 2 was revealed by serial contrast-enhanced CT scannings. Film A was taken 2 weeks before the first injection of the autologous T cell line, B was taken just after the 3rd injection (at 3 weeks after the initiation of the therapy), C: after the 7th injection (at 5 weeks), D: after the 9th injection (at 7 weeks), E: after the 11th injection (at 9 weeks), and F was taken after 5 weeks after the final (13th) injection, that was 16 weeks after the first injection, respectively.

formance status according to the Karnofsky rating system [22]. Toxicities and complications of the therapy were carefully evaluated; general status and neurological signs and symptoms were attentively observed, hematological workups and serum chemistries were checked weekly, and immunological status was monitered biweekly. From 4 weeks prior to the immunotherapy, and during the course of the therapy, other treatments for cancer were not performed.

Table 6 summarizes the result of the immunotherapy. Tumor regressions were observed in 2 patients out of 5, in case 2 and in case 5. There were no effects in other 3 patients. In case 2 of a thalamic GM patient, serial CT scannings (Fig. 2) revealed the gradual shrinkage of the tumor which was noted after the 3rd injection of the cells (Fig. 2B). Tumor size continued to regress even after the cessation of the immunotherapy, and became less than 50% of the original size in diameter on the CT film taken at 5 weeks after the final (13th) administration of the cells (Fig. 2F). However, the residual tumor started to regrow at 20 weeks after the cessation of the immunotherapy. Although he received a high dose dexamethasone therapy combined with irradiation, he expired 42 weeks after the cessation of the immunotherapy, 16 months after his clinical onset.

In case 5 of malignant mixed glioma, the postoperative thin and shell-shaped residual tumor became equivocal on CT films taken at 4 weeks after the cessation of the immunotherapy. Now it has passed 104 weeks after the final injection of autologous T cell line, he is full of his activity in his occupation without any neural deficits.

Other 3 cases of GM patients, 2 recurrent cases and one primary case, did not respond to the immunotherapy, and died at the early period after the therapy (Table 6). Particularly in 2 patients of recurrent GM, large postoperative tumor masses continued to enlarge in spite of the repeated administrations of autologous T cell line that effectively killed the tumor in vitro. There might be several reasons for the failure of the therapy in these patients. One was that the injected effector cells were too small to interfere with the tumor growth of such a large bulk tumor. Second, specific CTL was not sufficient for the effective tumor-rejection, and some other factors such as helper T cell might be required. Third, since the specificity of the CTL was assessed on the glioma cells in vitro, it was possible that the antigenic determinants might be changed in tumor cells in vitro. Thus, CTLs might not react with tumors in vivo. Further examinations are needed to get a conclusion.

In all cases, autologous T cell line containing specific CTL was safely administered locally into tumor-bed via an Ommaya reservoir with no complications nor toxicities. It should be stressed that case 5 patient is still alive after 2 years from the therapy with no neurological deficits, which indicates that this immunotherapy might be free from late occurring complications.

Adoptive immunotherapy is one of the most promising therapies for cancers. In animal models, an adoptive immunotherapy has been well established to be a curative procedure for experimental tumors [1–5]. However, in human system, an adoptive immunotherapy has been hampered by a difficulty to obtain a large amount of tumor-specific T cells. Since nonspecifically activated lymphocytes were known to kill various tumor cells, administrations of LAK cells [6, 7, 9], PAK cells [7, 8], or AAK cells [10] were done in patients with neoplasms to show ambiguous effects on tumors. Recently, Rosenberg's group started to administer LAK cells in the combination of rIL-2 infusions, and reported that the combination therapy was effective on certain tumors [11]. In this communication, we added another strategy to get effector cells for the adoptive immunotherapy; induce the tumor specific CTL by a mixed lymphocyte-tumor culture, and expand the tumor-reactive cells with IL-2. Whether such a strategy is also valid in other tumor systems should be clarified in future work.

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