Induction of tumor-specific cytotoxic T lymphocytes by tumor necrosis factor- α (TNF α) in cancer patients

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Cytotoxic T lymphocytes (CTL) were induced from the peripheral blood mononuclear cells of 6 cancer patients using tumor necrosis factor- α (TNF α). Initially, TNF α (100 and 1,000 U/ml) was added at the beginning of mixed culture with tumor lymphocytes for 3 days. Then, TNF α was removed and the cells were stimulated with an immobilized anti-CD3 monoclonal antibody (1 μ g/ml) and interleukin-2 (1,000 U/ml) for 3-7 days.

Subsequently, the anti-CD3 antibody was removed, and the cells were incubated with 500 U/ml of interleukin-2. Cells from 2 patients showed autologous tumor-killing activity before incubation with TNF α , and this activity was unchanged by TNF α . In 2 of the 4 patients without pre-existing cytotoxic T lymphocyte (CTL) activity, there was an increase of this activity after incubation with TNF α . Both CTL activity and CTL proliferation increased in accordance with the TNF α concentration.

Flow cytometric analysis revealed an increase of CD8⁺Leu15⁺ cells. However, the increase in cytotoxic activity also occurred when allogeneic cancer cells were identified as target cells, suggesting that TNF α did not increase tumor specificity although it increased autologous tumor-killing activity. Since a high CTL activity specific to autologous tumor cells is necessary for successful CTL therapy, this subject requires further investigation.

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It has been believed that cytotoxic T lymphocytes (CTL) are induced in about half of cancer patients by mixed culture of peripheral blood mononuclear cells (PBMC) and autologous tumor cells. However, blood levels of various immunosuppressors such as transforming growth factor- β (TGF β) increase as cancer progresses, leading to gradually increasing difficulty in achieving the induction of CTL¹⁾. The existence of tumor-specific suppressor T cells has been suggested to account for this immunosuppressive activity²⁾.

Accordingly, when CTL are not inducible by mixed culture, induction has been attempted with intravenous injection of 100-300mg of cyclophosphamide (Cy), which is believed to inhibit suppressor T cells^{3~7)}. However, no clearly effective method of *in vitro* induction has yet been established. Killer activating factor⁸⁾ and killer helper factor^{9,10)} have been reported as potential factors affecting CTL differentiation, but their roles have not been adequately elucidated in clinical or laboratory studies.

Tumor necrosis factor- α (TNF α) and various cytokines, such as interleukin (IL)-4, IL-5, IL-6, IL-7, and interferon-gamma, are believed to promote CTL differentiation^{11~17)}. TNF α was reported by Old et al.¹⁸⁾ as a substance causing tumor necrosis, and was subsequently shown to possess various actions^{19~22)}.

Table 1 HLA class I phenotypes of the tumor cell lines

Tumor cell line	HLA class I		
	A	B	C
Colon cancer	CW-2	A26/33	B35/44
			— / —
	CW-3	A11/24	B35/54
	TCM	A2/33	B44/51 (5)
	ACM	A2/11	B54/60
	UC-LM	A2/24	B7/54
Gastric cancer	OCM	A2/—	B35/48
			Cw3/—
	SS-90	A24/—	B51 (5)/62
	SS-92	A24/31	B51 (5)/61
	YGF	A2/—	B48/61
Breast cancer	GC071789	A26/33	B35/56
	NBM	A11/24	B35/48

Tumor cell lines were established in our laboratory. GC071789 was kindly provided by the Immunology Department of Kouchi Medical College.

In this study, we found that CD8⁺ CTL with a strong antitumor activity could be induced by addition of TNF α to mixed cultures containing patient PBMC and autologous tumor cells or HLA-matched allogeneic tumor cells. However, CTL were not necessarily induced in all patients with advanced cancer, and the proportion in whom CTL could be induced was still disappointingly low. Accordingly, this subject should be investigated further in the future.

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Table 2 Clinical profile of the patients

Patient	Age	Sex	PS	Cancer and status	Operation	Tumor cell line
1	55	male	1	colon cancer, Dukes C postoperative abdominal wall relapse	right hemicolectomy	OCM
2	54	female	2	breast cancer, Stage IIIA postoperative liver metastases (H ₂)	standard radical mastectomy	NBM
3	38	male	2	rectal cancer, Dukes B postoperative liver metastases (H ₂)	low anterior resection	UC-LM
4	76	male	3	rectal cancer, Dukes C inoperable	—	—
5	55	female	0	colon cancer, Dukes A postoperative	right hemicolectomy	CW-2
6	69	male	1	colon cancer, Dukes C postoperative	distal gastrectomy and transverse colon resection	ACM

Table 3 HLA class I phenotypes of the patients

Patient	HLA class I		
	A	B	C
1	A2/-	B35/48	Cw3/-
2	A11/24	B35/48	Cw3/-
3	A2/24	B7/54	Cw1/w7
4	A33/-	B44/-	-/-
5	A26/33	B35/44	-/-
6	A2/11	B54/60	Cw1/w7

Fig.1 Induction of tumor-specific CTL by TNF α and an immobilized anti-CD3 MAb plus IL-2

Material and methods

Tumor cell lines

Various cancer cell lines were established from fresh surgical specimens of gastric cancer, colorectal cancer and breast cancer or carcinomatous pleural effusion. These cancer cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY). HLA class I typing of the cancer cell lines was done by the standard microtoxicity test using peripheral blood lymphocytes (Table 1).

Cytokines

Recombinant human IL-2 was kindly provided by Shionogi Pharmaceutical Co. (Osaka, Japan) and recombinant TNF α was kindly provided by Dainippon Pharmaceutical Co. (Osaka, Japan).

Immobilized anti-CD3 monoclonal antibody

An anti-CD3 monoclonal antibody (100 μ l adjusted to 1 μ g/ml, COSMO BIO, Tokyo, Japan) was added to a 96-well flat-bottomed plate and incubated at 37°C in 5% CO₂ for 60 min, after which the plate was washed twice with RPMI 1640 and used for CTL induction.

Preparation of mononuclear cells and induction of tumor-specific cytotoxic T lymphocytes

Heparinized blood samples were collected from 6 cancer patients whose HLA class-I status was known (Tables 2, 3), and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation. Autologous cancer cells or allogeneic cancer cells with partial matching of the HLA-A locus were treated with mitomycin C (200 μ g/ml) for 60 min and added (at the ratio of 1 per 50 PBMC) to mixed cultures of lymphocytes and tumor cells in an incubator at 37°C in 5% CO₂.

The culture medium was RPMI 1640 supplemented with 100 units/ml of penicillin G, 100 g/ml of streptomycin, and 10% heat-inactivated fetal calf serum. At the beginning of culture, TNF α was added at concentrations of 100 U/ml and 1,000 U/ml. After incubation for 3 days, TNF α was removed, and 1 μ g/ml of the immobilized anti-CD3 antibody and 1,000 U/ml of IL-2 were added for stimulation. The culture plate was subsequently changed, and culture was

Fig. 2 Effects of $\text{TNF } \alpha$ on CTL generation

In patient 1, the stimulator and target cells were YGF cells (HLA-A2 and B48 matched), while CW-2 cells (HLA-A33 and B44 matched) were used in patient 4. In patients 2,3,5, and 6, stimulator and target cells were autologous tumor cells.

continued on TIL media (IBL, Takasaki, Japan) plus 1% AB serum containing 500 U/ml of IL-2 (Fig.1).

Cytotoxicity assay

A conventional 4-hour ^{51}Cr release assay was performed by mixing 1×10^4 ^{51}Cr -labeled tumor cells with 2×10^5 effector cells (effector/target ratio = 20) in 96-well round bottomed culture plates (Linbro, Flow Laboratories, Maclean, VA). The percent cytotoxicity was calculated using the following formula:

$$100 \times (\text{test cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm}).$$

All data were reported as the mean $\pm$ SD of triplicate experiments.

Statistical analysis

Results were analyzed using Student's *t*-test and differences in cytotoxicity were considered significant at $P < 0.05$.

Fluorescence-activated cell sorter (FACS) analysis

Cells were washed in Hank's balanced salt solution

Fig.3 Cytotoxic activity against allogeneic cancer cells

In patient 1, CW-3 and GC071789 cells (HLA-B35 matched) were used as the target cells. In patient 2, CW-3 and SS-90 cells (HLA-A2 matched) and TCM cells (HLA mismatched) were used.

containing 1% fetal calf serum and 0.2% sodium azide at 4°C . Cells (5×10^5 /tube) were stained with either the appropriate fluorescein isothiocyanate-or phycoerythrin-labeled monoclonal antibody (Becton Dickinson, Tokyo, Japan), incubated at 4°C for 45 min, washed twice, and resuspended in 0.5 ml of medium for FACS analysis.

Monoclonal antibodies

The following antibodies were obtained from Becton Dickinson: anti-Leu3a (anti-CD4), anti-Leu2a (anti-CD8), anti-Leu15, and anti-Leu8.

Results

CTL activity

CTL activity showed a distinct increase in 2 of the patients (patients 1 and 2). The increase of cytotoxic activity against tumor cells was dependent on the $\text{TNF } \alpha$ concentration and was highest at 1,000U/ml in both patients. When $\text{TNF } \alpha$ was added to the cells of 2 other patients who already showed a high cytotoxic activity, it had no influence on CTL activity (Fig.2).

Cytotoxicity for allogeneic cancer cells

The cytotoxic activity against cancer cells with mismatch at the HLA class- I locus was investigated in patients 1 and 2. In patient 1, there was a $\text{TNF } \alpha$ concentration-dependent increases of cytotoxic activity, which was similar to the response against autologous cancer cells. In patient 2, $\text{TNF } \alpha$ only showed cytotoxicity against autologous cancer cells and there was no increases of activity against allogeneic cancer cells with HLA class I mismatch (Fig.3).

Fig.4 Proliferative effect on CTL of $\text{TNF } \alpha$ with anti-CD3 MAb plus IL-2

Proliferation of activated lymphocytes from patient 1 was investigated.

○:control. ●: $\text{TNF } \alpha$ (100U/ml). △: $\text{TNF } \alpha$ (1,000U/ml)

CTL proliferation

Proliferation of activated lymphocytes was investigated in patient 1. The lymphocyte count increased along with the $\text{TNF } \alpha$ concentrations rising by 23- and 34-fold at 100 and 1,000 U/ml, respectively, while the count increased 17-fold after 3 weeks in cultures without $\text{TNF } \alpha$ (Fig.4).

Phenotype of induced CTL

The proportion of $\text{CD8}^+ \text{Leu15}^-$ cells was increased in patients 1 and 2, who showed an increase in antitumor activity with $\text{TNF } \alpha$. In patient 3, antitumor activity was not increased and the $\text{CD8}^+ \text{Leu15}^-$ cell count showed no increase. There was no obvious trend of the CD4^+ cell count (Table 4).

Discussion

In patients with progressive cancer, various immunosuppressive factors (including $\text{TGF } \beta$) secreted from macrophages and cancer cells are believed to contribute to the suppression of CTL differentiation, proliferation, and killing activity^{1,2}. PreCTL normally receive antigens from antigen-presenting cells, and express the IL-2 receptor prior to differentiation into CTL. In cancer patients, however, the lymphocyte response to IL-2 is known to decrease, and this is considered to be one cause of the difficulty in inducing CTL in patients with advanced cancer.

Table 4 Phenotypes of lymphocytes activated by $\text{TNF } \alpha$

		$\text{TNF } \alpha$ (-)	100U	1000U
Patient 1	$\text{CD8}^+ \text{Leu15}^-$	24.8%	40.4%	47.7%
	$\text{CD8}^+ \text{Leu15}^+$	0.6	3.7	3.4
	$\text{CD4}^+ \text{Leu8}^-$	0.8	2.1	4.2
	$\text{CD4}^+ \text{Leu8}^+$	73.4	52.4	24.5
Patient 2	$\text{CD8}^+ \text{Leu15}^-$	31.9	29.7	37.7
	$\text{CD8}^+ \text{Leu15}^+$	0.4	0.5	0.5
	$\text{CD4}^+ \text{Leu8}^-$	18.8	14.2	16.7
	$\text{CD4}^+ \text{Leu8}^+$	50.2	56.8	45.5
Patient 3	$\text{CD8}^+ \text{Leu15}^-$	3.7	7.7	7.3
	$\text{CD8}^+ \text{Leu15}^+$	0.2	0.4	0.4
	$\text{CD4}^+ \text{Leu8}^-$	8.5	22.7	12.1
	$\text{CD4}^+ \text{Leu8}^+$	38.0	68.1	34.0

$\text{CD8}^+ \text{Leu15}^-$ cells are cytotoxic T lymphocytes and $\text{CD4}^+ \text{Leu8}^+$ cells are helper T lymphocytes.

$\text{TNF } \alpha$ is mainly secreted by macrophages, and has various actions^{17,21,22}, most of which are antagonistic to those of $\text{TGF } \beta$ ²⁰, including the induction of IL-2 receptors. Fujimoto et al. recently reported that the action of killer activating factor is suppressed by an anti- $\text{TNF } \alpha$ antibody, and $\text{TNF } \alpha$ has been predicted to have an important role in the induction of CTL.

In the present study, $\text{TNF } \alpha$ was added at the beginning of mixed lymphocyte culture, the most important phases of CTL induction, in order to investigate its influence on CTL activity. There was an increase of CTL activity in 2 out of 4 patients, excluding 2 who already showed high autologous tumor-killing activity before the addition of $\text{TNF } \alpha$. The 2 patients in whom CTL activity was not increased had a far worse nutritional status than the other 2 patients, and this was considered to have contributed to our failure to induce CTL activity. The increase of CTL activity was dependent on the $\text{TNF } \alpha$ concentration, and this was also the case when allogeneic cancer cells were used as the target cells. These results suggested that $\text{TNF } \alpha$ did not increase tumor specificity although it increased autologous tumor-killing activity.

Our present results also suggested that part of the mechanism suppressing CTL induction can be blocked by $\text{TNF } \alpha$. $\text{TNF } \alpha$ enhances the expression of HLA class- I antigens by tumor cells¹⁹, and this may be one reason for the augmentation of CTL activity by addition of $\text{TNF } \alpha$ at the initiation of culture. However, immunosuppression cannot be reversed by $\text{TNF } \alpha$ alone, because there are many immunosuppressive factors (prostaglandins, $\text{TGF } \beta$, suppressor T cells, etc.) in patients with advanced cancer. This suggests that various therapeutic methods must be combined to achieve optimum CTL induction.

Cyclophosphamide is widely known to reverse the immunosuppression caused by suppressor T cells³⁻⁷. We

previously found that high CTL activity could be induced from PBMC isolated after administration of a relatively small dose of cyclophosphamide, even in patients who showed no CTL induction using a method similar to that of the present study²³). Immunomodulation by small doses of anticancer drugs has recently been attracting interest, and the combination of immunomodulation with CTL therapy may be useful.

CTL therapy was introduced by Fujimoto^{24,25}), and excellent results for various advanced cancers have been reported²⁶⁻²⁸). However, CTL activity decreases and the therapeutic response diminishes, as cancer progresses. We also treated several patients with advanced cancer, including patient 2, by CTL therapy. Liver metastases of gastric cancer were abolished by CTL injection via the hepatic artery, but the other results obtained were not encouraging. Therefore, the method of CTL induction must be improved and more potent killer cells with higher tumor specificity are needed to obtain better results. The present findings suggest that TNF α may potentiate CTL therapy in about half of the patients with advanced cancer, in whom CTL activity is not inducible by conventional methods. The effects of other cytokines should also be investigated in the future.

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