Human Tumor-infiltrating Lymphocytes Transfected with Tumor Necrosis Factor Gene Could Augment Cytotoxicity to Autologous Tumor Cells

Yoshinori Itoh, Yutaka Kohgo, Naoki Watanabe, Yuji Kanisawa, Sumio Sakamaki, Minoru Takahashi, Yasuo Hirayama, Hiroyuki Ono, Takeshi Himeno and Yoshiro Niitsu

Department of Internal Medicine (Section 4), Sapporo Medical College, South-1, West-16, Chuo-ku, Sapporo 060

Human tumor-infiltrating lymphocytes (TILs) derived from pleural or ascitic fluid were incubated with recombinant interleukin 2 and transfected with human tumor necrosis factor (TNF) α gene by the lipofection procedure. The resulting TILs secreted significant amounts of TNF in the culture supernatant and exhibited cytotoxicity against established cell lines, such as K562 and Daudi, and autologous tumor cells. The TNF gene-transfected TILs exhibited an augmented killing of autologous tumor cells.

Key words: Tumor-infiltrating lymphocyte — Tumor necrosis factor gene — Gene transfection

Tumor necrosis factor (TNF) is an antitumor cytokine with a wide variety of immunological actions.^{1,2} It exhibits its cytotoxic effect through direct and indirect actions. In addition to the direct killing of tumor cells, TNF can augment the cytotoxicity of natural killer (NK) cells and lymphokine-activated killer (LAK) cells,^{3,4} enhance the expression of HLA antigen⁵ and interleukin-2 (IL-2) receptors,⁴ and reinforce the induction of other cytokines such as interleukin 1 (IL-1)⁶ and granulocyte-macrophage stimulating factor (GM-CSF).⁷ Since mass production has provided large quantities of recombinant human TNF, clinical trials have been conducted in cancer patients at many institutions.⁸ However, the large doses of TNF needed to obtain a good clinical response were associated with intolerable side effects such as fever, hypotension, and thrombocytopenia. Therefore, methods to augment the effects of lower-dose TNF were investigated. It is known that LAK cells and tumor-infiltrating lymphocytes (TILs) are strongly cytotoxic to autologous tumor cells. It has also been reported that in animal models and clinical trials, TILs accumulate at local tumor sites after transfer and remain there for long periods.^{9,10} These characteristics of TNF and TILs can be utilized, if the antitumor activity of TILs is augmented when they are transferred into cancer-bearing hosts after being transfected with TNF expression vector. This process would increase the TNF concentration at the local tumor site. In the present study, we transfected the TNF expression vector, pcDV-TNF, into TILs and determined the resulting cytotoxicity against autologous tumor cells.

Human recombinant IL-2 (3.5×10^4 BRM units/mg protein) and recombinant TNF α (2.37×10^6 units/mg protein) were obtained from Takeda Pharmaceutical Co. Ltd., Osaka, and Asahi Chemical Industries Ltd., Tokyo,

respectively. K562 cells (human myelogenous leukemia cell line; NK-sensitive) and Daudi cells (Burkitt's lymphoma cell line; NK-resistant and LAK-sensitive) were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Flow Laboratories, McLean, VA). TILs were obtained from ascites of a gastric cancer patient (Case 1) and pleural effusion of two lung cancer patients (Cases 2 and 3). Pleural and ascitic fluid from lung or gastric cancers were layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) solution and centrifuged at 400g for 30 min. The resulting mononuclear cell layer containing TILs and autologous tumor cells was further separated by Percoll (Pharmacia) discontinuous gradient centrifugation. The TILs obtained were resuspended in 5×10^5 /ml of RPMI 1640 medium with 10% FCS and were cultured for 2 to 3 weeks in the presence of 2 units/ml of rIL-2. The autologous tumor cells were cryopreserved at -70°C until needed. The TNF expression vector, pcDV-TNF, has been described in detail elsewhere.¹¹ pcDV-TNF was derived from pSV2neo and human TNF coding region was inserted into it. pSV2neo was used as a control vector which encodes SV40 promoter and neomycin phosphotransferase, an intracellular enzyme that inactivates G418.¹² Gene transduction was performed using the lipofection method.¹³ After 1×10^6 TILs had been washed twice with Opti-MEM^R I reduced serum medium (GIBCO), they were resuspended in the same medium with 20 μg of vector DNA and 30 μg of LipofectinTM reagent (BRL, Gaithersburg, MD) and cultured for 4 to 8 h. Afterwards, 1.5 ml of RPMI 1640 medium with 20% FCS and 4 units/ml of rIL-2 were added, and TILs were cultured for an additional 18 h. Quantitation of TNF concentration in the supernatant of

the TIL culture was performed by enzyme-linked immunoassay using antihuman TNF monoclonal antibody.¹⁴⁾ The cytotoxic activity of TILs and transduced TILs was determined by ⁵¹Cr-release assay.¹⁵⁾ The assay period was 4 h when K562 cells and Daudi cells were used as targets and 12 h when autologous tumor cells were used as targets. The percent cytotoxicity was calcu-

Fig. 1. Effect of exogenous TNF on killing activity of TILs. Percentage cytotoxicity was determined by ⁵¹Cr release assay using K562, Daudi and autologous tumor cells as targets. □, K562; ▨, Daudi; ■, autologous tumor. Each bar represents the mean ± SD of three experiments. Data were analyzed statistically by the use of Student's *t* test. * *P* < 0.01. ** *P* < 0.05.

lated by using the following equation: % cytotoxicity = (experimental counts - spontaneous counts) × 100 / (total counts - spontaneous counts). The sensitivity to rhTNF of K562 cells, Daudi cells, and cryo-preserved autologous tumor cells was calculated from the results of the ⁵¹Cr-release assay. After adding various concentrations of TNF to 5 × 10³ tumor cells labeled with ⁵¹Cr (Daichi Radioisotope Laboratory, Tokyo), a 48 h culture was performed, and the radioactivity of the culture supernatant was counted.

We first examined the effect of exogenous TNF on the killing activity of TILs. TILs were cultured for 24 h in

Table I. TNF Concentration in Culture Supernatant of pcDV-TNF-gene-transfected TILs, pSV2neo-gene-transfected TILs, and Nontransfected TILs

	Incubation times for transfection (h)	TNF activity (unit/ml)		
		non-transfected TILs	pSV2neo-transfected TILs	pcDV-TNF-transfected TILs
Case 1 (Gastric cancer)	4	0.6	0.5	1.24
	6	0.42	0.28	0.66
	8	0.6	0.42	0.82
Case 2 (Lung cancer)	4	0.2 > ^{a)}	n.t. ^{b)}	1.13
	6	0.2 >	n.t.	1.31
	8	0.2 >	n.t.	1.37
Case 3 (Lung cancer)	6	0.2 >	n.t.	2.76

a) The lower limit of the assay system is 0.2 unit/ml.

b) Not tested.

Table II. Cytotoxic Activity and Spectrum of Exogenous TNF, Nontransfected TILs, and TNF Gene-transfected TILs against K562, Daudi, and Autologous Tumor Cells

		K562	Daudi	Autologous tumor
TNF	0.1 unit/ml	12.0 ± 2.1 ^{a)}	8.0 ± 1.3	1.8 ± 0.9
	1 unit/ml	15.7 ± 4.1	10.3 ± 3.5	2.6 ± 1.5
	10 units/ml	20.2 ± 5.4	17.5 ± 3.2	1.3 ± 0.8
	100 units/ml	33.4 ± 3.3	27.2 ± 4.6	1.9 ± 1.1
	1000 units/ml	42.7 ± 4.6	36.0 ± 5.4	1.2 ± 0.5
Nontransfected TILs	Case 1 (E/T ^{b)} = 20)	17.2 ± 2.4	3.3 ± 1.6	n.t. ^{c)}
	Case 2 (E/T = 20)	10.0 ± 1.8	10.3 ± 1.1	15.4 ± 3.7
	Case 3 (E/T = 2)	10.9 ± 2.1	19.6 ± 1.5	1.8 ± 1.9
pcDV-TNF-transfected TILs	Case 1 (E/T = 20)	27.0 ± 5.4 ^{d)}	5.1 ± 2.4	n.t.
	Case 2 (E/T = 20)	18.9 ± 0.7 ^{d)}	8.5 ± 1.4	41.0 ± 6.3 ^{d)}
	Case 3 (E/T = 2)	28.6 ± 0.9 ^{d)}	21.4 ± 0.8	25.0 ± 4.6 ^{d)}

a) Means ± SD.

b) Effector/target ratio.

c) Not tested.

d) Significantly different from cytotoxicity of nontransfected TILs (*P* < 0.01) by Student's *t* test.

the presence of 2 units/ml of rIL-2 and various concentrations of TNF, and the cytotoxicity to K562 cells, Daudi cells, and autologous tumor cells was measured. As shown in Fig. 1, the antitumor activity of TILs was increased by the concurrent use of 0.1 to 100 units/ml of TNF and rIL-2. TNF had the greatest effect at 10 units/ml concentration. Because the addition of exogenous TNF reinforced the TIL activity, we attempted the transduction of TNF gene into TILs. After a pcDV-TNF vector was inserted into the TILs, 0.66 to 2.76 units/ml of TNF was found in the culture supernatant at 18 h (Table I). Only a trace of TNF (0.6 unit/ml, maximum) was found in the culture supernatant of normal TILs and those transfected with the pSV2neo vector. The expression of TNF in TILs transfected with the pcDV-TNF vector reached a peak 18 to 20 h after gene induction and decreased to the control level after 48 h (data not shown). Table II shows the extent and spectrum of cytotoxicity of exogenous TNF, TILs, and TNF gene-transfected TILs against K562, Daudi, and autologous tumor cells. Before calculating the antitumor activity of the genetically transformed TILs, we examined the TNF sensitivity of the target tumor cells. The percent cytotoxicity of K562 cells and Daudi cells in the presence of 1000 units/ml of TNF was 42.7% and 36.0%, respectively, showing a moderate sensitivity to TNF. None of the autologous tumor cells showed sensitivity to TNF. The cytotoxicity of TNF gene-transfected TILs toward K562 cells was $27.0 \pm 5.4\%$ (Case 1) $18.9 \pm 0.7\%$ (Case 2) and $28.6 \pm 0.9\%$ (Case 3), which represented an increase in cytotoxicity of approximately 1.6- to 1.9-fold compared with non-transfected TILs ($17.2 \pm 2.4\%$ in Case 1, $10.0 \pm 1.8\%$ in Case 2 and $10.9 \pm 2.1\%$ in Case 3). Although Daudi cell have the same degree of TNF sensitivity as K562 cells, there was no reinforcement of the activity of TILs by pcDV-TNF vector transduction. It is noteworthy that the cytotoxicity of pcDV-TNF-inserted TILs against autologous tumor cells was approximately 2.6 times that of the nontransfected TILs at an effector/target (E/T) ratio of 20 ($41.0 \pm 6.3\%$ compared with $15.4 \pm 3.7\%$ in Case 2, $P < 0.01$), although the autologous tumor cells were insensitive to TNF. Reinforcement of the activity of TILs by TNF gene induction was especially marked at a low E/T ratio ($25.0 \pm 4.6\%$ compared with $1.8 \pm 1.9\%$ in Case 3, $P < 0.01$).

In this study, we investigated the generation of TNF by TILs after the insertion of the TNF-expressing vector. Following the TNF gene transduction, a significant amount of TNF was found in the culture supernatant of TILs when compared with that of the nontransduced cells, suggesting that TNF-producing TILs can be effective carriers for TNF to distant tumor sites. In addition, the reinforcement of cytotoxicity was seen in relation to

K562 cells and autologous tumor cells with pcDV-TNF-bearing TILs. Since the reinforcement of cytotoxic activity was not seen in Daudi cells, which have virtually the same degree of TNF sensitivity as K562 cells, and since such an action was seen in autologous tumor cells, which are TNF-insensitive, it appears that unknown factor(s) such as cytokines produced by TNF-gene-transfected TILs may influence the role of TNF. It is possible that increase in or clustering of TNF receptors, augmentation of pinocytosis, action of perforin, change of permeability of the cell membrane, etc. in the target cells are related to the influx of TNF. The sensitivity of target cells to the factors causing the above effects may be different among K562, Daudi and autologous tumor cells. Concerning the mechanism of the reinforcement of lymphocyte cytotoxicity by TNF, at least two mechanisms of action have to be considered. One possibility is that endogenous TNF plays an important part in the induction and maintenance of the cytotoxic activity of lymphocytes. Recently, Blay *et al.* reported that TNF activity was found in culture supernatant when large granular lymphocytes were incubated with IL-2 and that the induction of LAK activity by IL-2 was inhibited by anti-TNF antibodies,¹⁶ suggesting an important role of TNF in lymphocyte activation. From the clinical point of view, however, it is not easy to maintain the IL-2 concentration and endogenous TNF mRNA expression of TILs at local tumor sites: 1×10^2 units/ml of IL-2 was necessary for endogenous TNF induction in the study by Blay *et al.* Accordingly, the genetic induction of TNF production by TILs to make TILs capable of continuously supplying endogenous TNF seems to be a worthwhile approach. Another possible explanation for cytotoxicity reinforcement by TNF gene transduction is an increase in the adhesiveness of TILs to tumor cells due to a TNF-promoted increase in MHC antigen expression of tumor cells.⁵ However, this mechanism was not believed to have a major role in our study because of the relatively short time needed for TILs to have a cytotoxic effect (4 to 12 h) compared with the later expression peak of MHC antigen in tumor cells (approximately 48 to 72 h after treatment with TNF).¹⁷ Many aspects of the mechanism of reinforcement of the cytotoxic activity of lymphocytes by TNF remain to be elucidated, but TNF gene-transfected TILs should be valuable in the development of new approaches to immunotherapy.

This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan to Y.K. (No. 1-4) and for Scientific Research from the Ministry of Education, Science and Culture of Japan to Y.N. (No. 03152108) and Y.K. (No. 03770392).

(Received May 13, 1991/Accepted August 5, 1991)

REFERENCES

- 1) Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA*, **72**, 3666-3670 (1975).
- 2) Watanabe, N., Niitsu, Y., Neda, H., Sone, H., Yamauchi, N., Umetsu, T. and Urushizaki, I. Antitumor effect of tumor necrosis factor against various primarily cultured human cancer cells. *Jpn. J. Cancer Res.*, **76**, 1115-1119 (1985).
- 3) Ostensen, M. E., Theile, D. L. and Lipsky, P. E. Tumor necrosis factor- α enhanced cytolytic activity of human natural killer cells. *J. Immunol.*, **138**, 4185-4191 (1987).
- 4) Ouen-Schaub, L. B., Gutterman, J. U. and Grimm, E. A. Synergy of tumor necrosis factor and interleukin 2 in the activation of human cytotoxic lymphocytes: effect of tumor necrosis factor α and interleukin 2 in the generation of human lymphokine-activated killer cell cytotoxicity. *Cancer Res.*, **48**, 788-792 (1988).
- 5) Pfizenmaier, K., Scheurich, P., Schluter, C. and Krönke, M. Tumor necrosis factor enhances HLA-A, B, C, and HLA-DR gene expression in human tumor cells. *J. Immunol.*, **138**, 975-980 (1987).
- 6) Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A. and O'Connor, J. V. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin-1. *J. Exp. Med.*, **163**, 1433-1450 (1986).
- 7) Munker, R., Gasson, J., Ogawa, M. and Koffler, H. P. Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor. *Nature*, **323**, 79-82 (1986).
- 8) Kimura, K., Taguchi, T., Urushizaki, I., Ohno, R., Abe, O., Furue, H., Hattori, T., Ichihashi, H., Inoguchi, K., Majima, H., Niitani, H., Ota, K., Saito, T., Suga, S. and The α_1 -TNF Cooperative Study Group. Phase I study of recombinant human tumor necrosis factor. *Cancer Chemother. Pharmacol.*, **20**, 223-229 (1987).
- 9) Fisher, B., Packard, B. S., Read, E. J., Carrasquillo, J. A., Carter, C. S., Topalian, S. L., Yang, J. C., Yolles, P., Larson, S. M. and Rosenberg, S. A. Tumor localization of adoptively transferred indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. *J. Clin. Oncol.*, **7**, 250-261 (1989).
- 10) Rosenberg, S. A., Aebersold, P. M., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., Merino, M. J., Culver, K., Miller, A. D., Blaese, R. M., Anderson, W. F. and Kasid, A. Gene transfection into humans — immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transfection. *N. Engl. J. Med.*, **323**, 570-578 (1990).
- 11) Himeno, T., Watanabe, N., Yamauchi, N., Maeda, M., Tsuji, Y., Okamoto, T., Neda, H. and Niitsu, Y. Expression of endogenous tumor necrosis factor as a protective protein against the cytotoxicity of exogenous tumor necrosis factor. *Cancer Res.*, **50**, 4941-4945 (1990).
- 12) Southern, P. J. and Berg, P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.*, **1**, 327-341 (1982).
- 13) Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA*, **84**, 7413-7417 (1987).
- 14) Yamazaki, S., Onishi, E., Enami, K., Natori, K., Kohase, M., Sakamoto, H., Tanouchi, M. and Hayashi, H. Proposal of standardized method and reference for assaying recombinant human tumor necrosis factor. *Jpn. J. Med. Sci. Biol.*, **39**, 105-115 (1988).
- 15) Kadish, S. A., Doyle, T. A., Steinhauer, H. E. and Ghossein, N. A. Natural cytotoxicity and interferon production in human cancer. Efficient natural killer activity and normal interferon production in patients with advanced disease. *J. Immunol.*, **127**, 1817-1822 (1981).
- 16) Blay, J. Y., Bertoglio, J., Fradelizi, D. and Chouaib, S. Functional interactions of IL-2 and TNF in the differentiation of LGL into LAK effectors. *Int. J. Cancer*, **44**, 598-604 (1989).
- 17) Stotter, E., Wiebke, E., Tomita, S., Beldegrun, A., Topalian, S., Rosenberg, S. A. and Lotze, M. T. Cytokines alter target cell susceptibility to lysis. II. Evaluation of tumor infiltrating lymphocytes. *J. Immunol.*, **142**, 1767-1773 (1989).