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Inhibition of Tumor Necrosis Factor-α and -β Secretion by Lymphokine Activated Killer Cells by Transforming Growth Factor-β

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Transforming growth factor-β (TGF-β) has a variety of immunosuppressive properties. We investigated the effect of TGF-β secreted by glioblastoma (T98G) cells on the secretion of tumor necrosis factor-α and -β (TNFs) by lymphokine activated killer (LAK) cells stimulated with tumor cells. The supernatant from T98G cells was preincubated with anti-TGF-β1 and -β2 neutralizing antibodies or untreated, and added to a coculture of LAK and Daudi cells. The neutralizing antibodies were added to LAK/Daudi and LAK culture, and natural human TGF-β1 and recombinant human TGF-β2 were also added to the LAK/Daudi culture. LAK cells were also cultured with T98G cells, of which the supernatant contained both active and latent forms of TGF-β1 and TGF-β2, and the neutralizing antibodies were added to the coculture. TNFs activity in the supernatants from LAK/Daudi cultures was examined by a specific bioassay. Addition of the supernatant from T98G cells to LAK/Daudi culture resulted in the inhibition of TNFs secretion by LAK cells. The inhibition was abrogated by the pretreatment of the supernatants with the anti-TGF-β antibodies. Addition of TGF-β1 and TGF-β2 to LAK/Daudi culture inhibited TNFs secretion by LAK cells in a dose-dependent manner. Addition of anti-TGF-β antibodies to LAK culture resulted in an increase of TNFs secretion. These results suggest that, if tumor cells have the capacity to convert TGF-β from a latent to an active form, the active TGF-β suppresses TNFs secretion by LAK cells stimulated with the tumor cells, and that TGF-β secreted and activated by glioblastoma cells suppresses the propagation of immune reaction by inhibiting TNFs secretion by activated lymphocytes adjacent to tumor cells.

Key words: Glioblastoma — Transforming growth factor-β — Lymphokine activated killer cell — Tumor necrosis factor — Immunosuppression

Patients with malignant gliomas have been treated with lymphokine activated killer (LAK) cells.[1-3] Although LAK therapy showed some efficacy on malignant gliomas, LAK therapy *in vivo* has been less effective than expected from *in vitro* studies. Our previous studies[4, 5] showed that LAK cells secreted tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), and interferon-γ, and the secretion increased when LAK cells were cultured with non-gliomatous tumor cells. Malignant glioma cells, however, abrogated the increase of cytokine secretion by LAK cells. We also showed that glioblastoma cells secreted both active and latent forms of transforming growth factor-β1 (TGF-β1) and TGF-β2[6] and suggested that TGF-β may inhibit the cytokine secretion from LAK cells and other immune effector cells, leading to suppression of the local immune reaction to tumor cells.

TGF-β, a peptide first identified on the basis of its ability to cause phenotypic transformation of rat fibroblasts,[7] has multiple effects on both normal and transformed cells.[8] Three distinct TGF-β polypeptides, TGF-β1, TGF-β2, and TGF-β3, have been identified in humans.[9-12] Recent studies have shown that TGF-β acts as a growth-inhibitory factor,[8, 13] and have defined a variety of immunoregulatory properties of TGF-β, including inhibition of T cell proliferation,[14] interleukin-2 (IL-2) receptor induction,[14] cytokine production,[15, 16] natural killer cell activity,[17] cytotoxic T cell development,[18, 19] LAK cell generation,[15, 18] and production of tumor-infiltrating lymphocytes.[20] Most cells secrete TGF-β in a latent form.[8] TGF-β exerts its biological functions after conversion to an active form.[13] Our previous studies[6] suggested that glioblastoma cells had the capacity to convert TGF-β from a latent to an active form.

LAK cells can kill tumor cells and, at the same time, secrete cytotoxic or cytostatic cytokines. On the other hand, most tumor cells may secrete and activate a latent form of TGF-β. How TGF-β affects the cytokine secretion by LAK cells stimulated with tumor cells is not well understood. In the present study, we investigated the effect of TGF-β secreted by glioblastoma cells on the secretion of TNF-α and TNF-β (TNFs) by LAK cells stimulated with tumor cells, and found that TGF-β suppresses TNFs secretion by LAK cells.

MATERIALS AND METHODS

Cell lines Human glioblastoma cell line (T98G) and Daudi cell line (human B tumor cell line derived from a patient with Burkitt's lymphoma) were obtained from the Japanese Cancer Research Resources Bank (Tokyo). L.P3 mouse fibroblast cell line, which is sensitive to both TNF-α and -β, was donated by Dr. Y. Miyagawa, Department of Microbiology, Yamanashi Medical University. These tumor lines were maintained in RPMI1640 medium supplemented with 2 m*M* glutamine, 100 μg/ml kanamycin, 0.05 m*M* 2-mercaptoethanol, and 10% fetal bovine serum.

Induction of LAK cells LAK cells were induced as described elsewhere.[4] Briefly, peripheral blood mononuclear cells were collected from the heparinized peripheral blood of normal healthy donors by centrifugation on a Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient. Peripheral blood mononuclear cells were cultured at 2×10^6 cells/ml in RPMI1640 medium with 10% fetal bovine serum and 2000 JRU (Japan reference unit)/ml of IL-2 (Takeda Pharmaceutical Co., Ltd., Osaka). After culture for 3 to 5 days, LAK cells were collected.

Collection of conditioned medium Subconfluent T98G cells cultured in tissue culture flasks (25 cm^2; Becton Dickinson, Franklin Lakes, NJ, USA) were washed three times with phosphate-buffered saline and cultured in 3 ml of serum-free RPMI1640 medium with or without 1 μg/ml of indomethacin (Sigma Chemical Co., St. Louis, MO, USA). After 24 h, the supernatants were collected, filtered (0.22 μm; Millipore Ltd., Tokyo), and stored at -20°C until use. Previous studies[6] indicated that the supernatants contained both active and latent forms of TGF-β1 and TGF-β2.

LAK cell culture with Daudi cells LAK cells were adjusted to a concentration of 1×10^6 cells/ml in complete medium with 5% fetal bovine serum and incubated with Daudi cells at a LAK/Daudi ratio of 10:1 at 37°C under 5% CO_2 in 24-well plates (Corning, NY, USA). LAK/Daudi cultures were supplemented with 1) 300 μl of the T98G supernatant treated with or without indomethacin, 2) 600 μl of the T98G supernatant preincubated with anti-TGF-β1 (R&D Systems, Minneapolis, MN, USA) and anti-TGF-β2 (R&D Systems) neutralizing antibodies (final concentration, 6 μg/ml), 3) indomethacin (final concentration 0.3 μg/ml), 4) anti-TGF-β1 and anti-TGF-β2 antibodies (final concentration of each antibody, 6 μg/ml), 5) 1 ng, 100 pg, 10 pg/ml of natural human TGF-β1 (Genzyme, Cambridge, MA, USA) or recombinant human TGF-β2 (Austral Biologicals, San Ramon, CA, USA), respectively, and 6) chicken immunoglobulin G (Cappel, Durham, NC, USA) or rabbit immunoglobulin G (Cappel) (final concentration, 6 μg/ml), giving 1 ml total volume. LAK cells were incubated with anti-TGF-β1 and anti-TGF-β2 antibodies (final concentration of each antibody, 6 μg/ml) and chicken immunoglobulin G or rabbit immunoglobulin G (final concentration, 6 μg/ml). After 16 to 18 h, the culture supernatants were harvested, filtered, and stored at -20°C until use.

Preliminary studies indicated that at least 10 μg/ml of anti-TGF-β1 or anti-TGF-β2 antibodies completely neutralized 2.5 ng/ml of natural human TGF-β1 or recombinant TGF-β2, respectively, and that anti-TGF-β1 antibody did not neutralize TGF-β2 and *vice versa*. The T98G supernatant was preincubated with anti-TGF-β1 (10 μg/ml) and anti-TGF-β2 (10 μg/ml) antibodies for 1 h at room temperature.

LAK cell culture with T98G cells LAK cells were adjusted to a concentration of 1×10^6 cells/ml in complete medium with 5% fetal bovine serum and incubated with T98G cells at a LAK/T98G ratio of 10:1 at 37°C under 5% CO_2 in 24-well plates (Corning). LAK/T98G cultures were supplemented with anti-TGF-β1 and/or anti-TGF-β2 antibodies. After 16 to 18 h, the culture supernatants were harvested, filtered, and stored at -20°C until use.

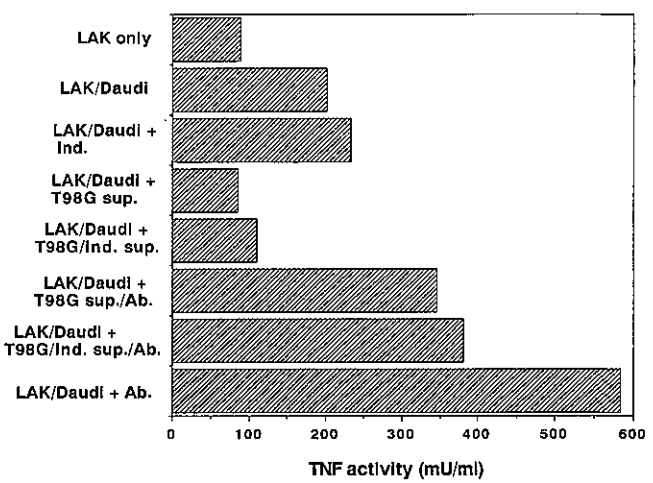

Fig. 1. Effect of T98G supernatant on TNF-α and TNF-β secretion by LAK cells stimulated with Daudi cells. Cocultures of LAK and Daudi cells were supplemented with the T98G supernatant, giving 1 ml total volume. Ab. = anti-TGF-β1 (20 μg/ml) and β2 (20 μg/ml) antibodies, Ind. = indomethacin (0.3 μg/ml), T98G sup. = T98G supernatant, T98G/Ind. sup. = culture supernatant from T98G treated with indomethacin (1 μg/ml), T98G sup./Ab. = T98G supernatant preincubated with anti-TGF-β1 and β2 antibodies, T98G/Ind. sup./Ab. = culture supernatant from T98G treated with indomethacin (1 μg/ml) was preincubated with anti-TGF-β1 and β2 antibodies. TNF activity includes TNF-α and TNF-β activities. The figure represents three experiments.

Measurement of TNF activity The assay for TNF activity in culture supernatants was described previously.[4] Briefly, serial two-fold dilutions of the supernatants were done in duplicate and were added to 5×10^4 L.P3 cells per well in 96-well flat-bottomed microtiter plates (Corning) in the presence of 1 μg/ml actinomycin D (Sigma Chemical Co.). After incubation for 16 h at 37°C, plates were stained with 0.5% crystal violet in 20% methanol for 5 min and washed thoroughly. The dye was dissolved in 50% ethanol and plates were read on a Titertek Multiskan (Flow Laboratories Inc., Helsinki, Finland). Human recombinant TNF-β (donated by Kanegafuchi Chemical Industry, Hyogo), with a specific activity of 3.2×10^5 U/mg protein, was used as an internal standard. The supernatants were preincubated with anti-TNF-α (5 μg/ml; R&D Systems) and anti-TNF-β (10 μg/ml; R&D Systems) to specify the activity. In preliminary studies, the antibodies completely neutralized 1 ng/ml of recombinant TNF-α (Dainippon Pharmaceutical Co., Ltd., Osaka) and 5 ng/ml of recombinant TNF-β, respectively. The dilution causing lysis of 50% of the cells was determined and the activity of TNF (mU/ml) was calculated from the standard dilution curve. The activity was expressed as a total activity including TNF-α and TNF-β. The reproducibility of this bioassay was reasonable, with a coefficient of variation between assays of 23–30% depending on dilution.

RESULTS

Inhibition of TNFs secretion by TGF-β in LAK/Daudi culture LAK cells constitutionally secreted TNFs, and Daudi cell stimulation resulted in the increase of TNFs secretion by LAK cells (Fig. 1). Increased TNFs secretion by LAK cells was suppressed by addition of T98G supernatant in LAK/Daudi culture. When LAK/Daudi culture was supplemented with T98G supernatant pretreated with anti-TGF-β1 and anti-TGF-β2 neutralizing antibodies or with the antibodies alone, TNFs secretion by LAK cells increased more than in LAK/Daudi culture. Addition of indomethacin to the cultures resulted in only a small increase of TNFs secretion by LAK cells. TNF activity in the supernatants from LAK/Daudi cultures was due to both TNF-α and TNF-β (Table I). TNF activity in all supernatants tested was completely neutralized with both anti-TNF-α and anti-TNF-β antibodies. In the preliminary studies, TGF-β and TNF activities in the culture supernatant of Daudi cells were not detected with the specific bioassays. Indomethacin did not affect the viability of L.P3 in the TNF bioassay.

To examine the effect of anti-TGF-β neutralizing antibody, the antibodies were added to LAK culture (Fig. 2). TNFs secretion by LAK cells increased with the addition of anti-TGF-β1 (chicken immunoglobulin G) and/or anti-TGF-β2 (rabbit immunoglobulin G) neutralizing antibodies, while chicken and rabbit immunoglobulin G did not affect TNFs secretion by LAK cells.

Natural human TGF-β1 and recombinant human TGF-β2 suppressed TNFs secretion by LAK cells in LAK/Daudi culture in a dose-related manner (Fig. 3). Addition of 100 pg/ml of TGF-β1 or TGF-β2 resulted in more than 50% inhibition of TNFs secretion by LAK cells. In preliminary studies, the T98G supernatant was

Table I. TNF-α and TNF-β Activities in the Supernatants from Cocultures of LAK and Tumor Cells

Supernatant	Cytotoxic activity[a] (mU/ml)			
	Treatment of supernatants[b] with			
	medium[c]	anti-TNF-α[d]	anti-TNF-β[e]	anti-TNF-α/β
LAK/Daudi				
Experiment 1	79	31	38	UD
Experiment 2	202	126	76	UD
LAK/T98G	42	19	34	UD

Daudi = Burkitt's lymphoma cell line, T98G = glioblastoma cell line, UD = undetectable.
a) Cytotoxic activity calculated from the standard dilution curve using human recombinant TNF-β.
b) Supernatants from cocultures of LAK and tumor cells.
c) RPMI1640 medium with 10% fetal bovine serum.
d) Anti-TNF-α antibody (5 μg/ml).
e) Anti-TNF-β antibody (10 μg/ml).

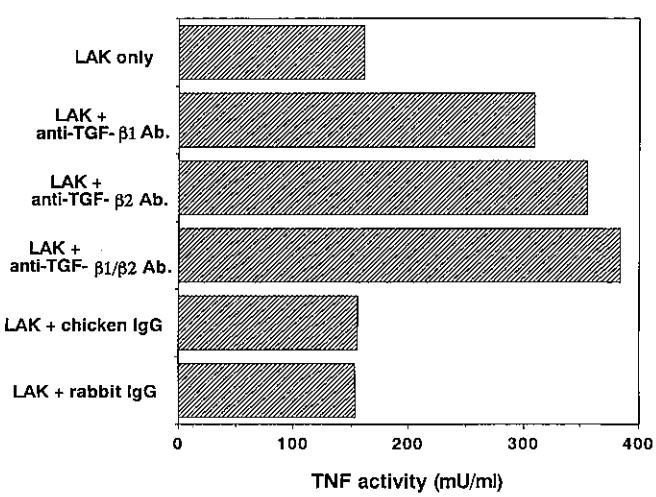

Fig. 2. Effect of anti-TGF-β1 and anti-TGF-β2 antibodies on TNF-α and TNF-β secretion by LAK cells. Anti-TGF-β1 (chicken IgG, 6 μg/ml) and/or anti-TGF-β2 (rabbit IgG, 6 μg/ml) antibodies were added to LAK culture. Chicken IgG or rabbit IgG (6 μg/ml of final concentration) was added to LAK culture. Ab. = antibody. The figure represents two experiments.

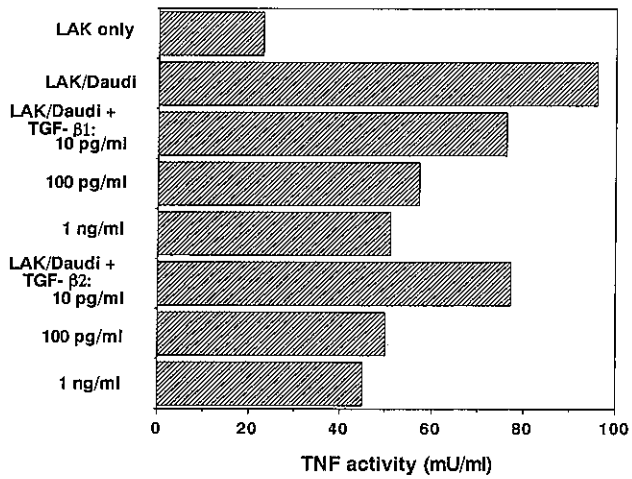

Fig. 3. Effect of TGF-β1 and TGF-β2 on TNF-α and TNF-β secretion by LAK cells. Natural human TGF-β1 or recombinant human TGF-β2 was added to LAK/Daudi culture. The figure represents three experiments.

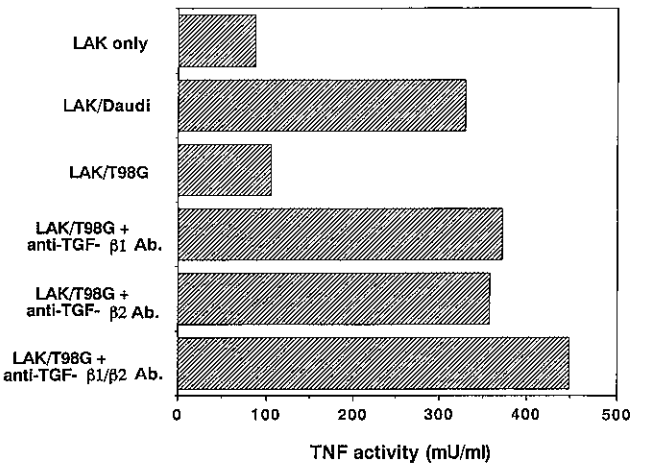

Fig. 4. Effect of anti-TGF-β1 and anti-TGF-β2 antibodies on TNF-α and TNF-β secretion by LAK cells stimulated with T98G glioblastoma cells. Ab. = antibody. The figure represents two experiments.

found to contain more than 100 pg/ml of TGF-β1 and TGF-β2 in several assays where recombinant human TGF-β2 was used as an internal standard (data not shown). TGF-β1 and TGF-β2 did not affect the viability of L.P3 cells in the TNF bioassay.

Inhibition of LAK cell TNFs secretion by T98G cells

TNFs secretion by LAK cells was not stimulated with T98G cells in contrast to the stimulation with Daudi cells (Fig. 4). However, the addition of anti-TGF-β1 and/or anti-TGF-β2 antibodies in the LAK/T98G culture resulted in increase of TNFs secretion by LAK cells. The supernatant of T98G cells did not contain TNFs.

DISCUSSION

The present data indicate that, although LAK cells had the potential to secrete more TNFs when stimulated with tumor cells, TGF-β secreted and activated by glioblastoma cells inhibited TNFs secretion by LAK cells, and that exogenous TGF-β1 and TGF-β2 also exerted an inhibitory effect on TNFs secretion by LAK cells. These results confirm our previous suggestion that TGF-β secreted by malignant glioma cells might inhibit cytokine secretion by LAK cells.[4,5] This is consistent with previous reports that TGF-β inhibits secretion from peripheral blood mononuclear cells of various cytokines, including interferon-γ, TNF-α, and TNF-β.[15,16]

In the present study, we used glioblastoma cells, of which the supernatant contained both active and latent forms of TGF-β1 and TGF-β2,[6] in coculture of LAK and tumor cells. Some tumor cells have the capacity to convert TGF-β from a latent to an active form,[21] although the mechanism of the activation is not well understood. Patients bearing malignant tumors, including malignant gliomas, have been treated with LAK cells and the results showed some efficacy of the immunotherapy.[1-3,22,23] LAK cells injected into the patients may secrete more TNFs as well as kill tumor cells if the LAK cells contact the tumor cells. However, LAK cells may fail to secrete more TNFs when they contact tumor cells which possess a mechanism of converting latent TGF-β to the active form. This may suppress the propagation of cytokine signals and may result in a decrease of the efficacy of LAK therapy.

Patients with malignant gliomas manifest an immunosuppressive state of humoral and cell-mediated immunity.[24-27] Recent reports suggest the relevance of TGF-β to the immunosuppression in patients with malignant gliomas.[20,28] We found that glioblastoma cells *in vitro* secreted both TGF-β1 and TGF-β2, and had the capacity to convert TGF-β from a latent to an active form.[5] This suggests that active TGF-β may inhibit local immune reaction to malignant glioma cells by suppressing cytokine secretion from activated lymphocytes. It has been shown that activated lymphocytes express more high affinity TGF-β receptor than resting lymphocytes, TGF-β down-regulates the expression of IL-2 receptor on T lymphocytes, and activated T cells themselves synthesize and secrete TGF-β, which inhibits IL-2-dependent T cellproliferation.[14] These events suggest that, if activated lymphocytes infiltrate into tumor tissue, their proliferation or the capacity to secrete cytokine may be suppressed by TGF-β secreted by both tumor cells and lymphocytes themselves.

It has been shown that an active form of TGF-β is produced by cocultures of vascular endothelial cells and pericytes.[29] Furthermore, vascular endothelial cells have tissue plasminogen activator activity,[30] and a latent TGF-β secreted by tumor cells is converted to an active form by plasmin.[31] Most malignant tumor cells are rich in blood vessels. The conversion of TGF-β from a latent to an active form may occur around vascular endothelial cells, and the active form of TGF-β may enter the systemic blood circulation. TGF-β has also been shown to exert a preferential suppression of the $CD4^+$ helper T cell function.[32] This may contribute to the state of systemic immunosuppression.

In the present study, LAK cells secreted more TNFs when neutralizing anti-TGF-β1 and anti-TGF-β2 antibodies were added in the culture. This suggests that IL-2-stimulated lymphocytes themselves may secrete both TGF-β1 and TGF-β2, and possess the capacity to convert TGF-β1 and TGF-β2 from latent to active forms. This is consistent with previous reports that IL-2 stimulation of peripheral blood mononuclear cells resulted in an up-regulation of intracellular TGF-β mRNA and TGF-β biologic activity secreted in culture media,[14, 33] and that peripheral blood mononuclear cells secreted latent TGF-β1 on IL-2 stimulation and had the capacity to convert latent TGF-β into an active form.[34] The activation of latent TGF-β by LAK cells themselves may function as a negative feedback for the cytokine secretion.

In conclusion, the present study suggests that, if tumor cells have the capacity to convert TGF-β from a latent to an active form, LAK cells stimulated with the tumor cells fail to secrete more TNFs, and that TGF-β secreted by glioblastoma cells suppresses the propagation of the immune reaction by inhibiting TNFs secretion by activated lymphocytes adjacent to tumor cells. Further studies to elucidate the mechanisms of activation of latent TGF-β and of regulation of TGF-β secretion by malignant glioma cells may suggest a strategy for treating patients with malignant gliomas.

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