Secretion of Transforming Growth Factor- β 1 and - β 2 by Malignant Glioma Cells

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Abstract

The secretion of transforming growth factor- β (TGF- β), a growth inhibitory factor with immunosuppressive properties, was investigated in one glioblastoma cell line and seven surgically resected malignant glioma cells. Cultured cells from surgically resected tumors were examined immunohistochemically for glial fibrillary acidic protein (GFAP) and S-100 protein. The levels of TGF- β 1 and TGF- β 2 in culture supernatants from malignant glioma cells were determined by a specific bioassay using anti-TGF- β 1 and anti-TGF- β 2 antibodies. Two glioblastoma cell lines were cultured in the presence of TGF- β 1 or TGF- β 2 to assess the effect of TGF- β on the growth of glioblastoma cells. Cultured cells from surgically resected tumors were positive for both GFAP and S-100 protein. Both active and latent forms of TGF- β 1 and TGF- β 2 were detected in the culture supernatants from malignant gliomas, except in one patient with anaplastic astrocytoma which secreted only latent forms of TGF- β 1 and TGF- β 2. There was no statistical difference in the levels of TGF- β 1 and TGF- β 2 in glioblastomas and anaplastic astrocytomas. Neither TGF- β 1 nor TGF- β 2 affected the growth of glioblastoma cells. These findings suggest that most malignant glioma cells secrete both TGF- β 1 and TGF- β 2, can convert TGF- β from a latent to active form, and may suppress cytokine secretion by activated lymphocytes *in vivo* as well as *in vitro*.

Key words: malignant glioma, transforming growth factor- β , immunosuppression

Introduction

Transforming growth factor- β (TGF- β), a peptide first identified by its ability to cause phenotypic transformation of rat fibroblasts,⁴⁵⁾ has multiple effects on both normal and transformed cells.⁵²⁾ Recent studies have shown that TGF- β acts as a growth-inhibitory factor,^{51,56)} and have defined a variety of immunoregulatory properties of TGF- β , including inhibition of T and B cell proliferation,^{22,23)} interleukin-2 receptor induction,²³⁾ interleukin-1-induced thymocyte proliferation,⁴⁴⁾ cytokine production including interferon- γ , tumor necrosis factor- α (TNF- α), and TNF- β ,^{15,16)} natural killer cell activity,⁴⁸⁾ cytotoxic T cell development,^{20,43)} lymphokine-activated killer (LAK) cell generation,^{15,20,21)} and production of tumor-infiltrating lymphocytes.²⁶⁾ Three distinct TGF- β polypeptides, TGF- β 1, TGF- β 2, and TGF- β 3, have been identified in humans^{10,13,14,30)} and

all are secreted in a latent form.⁸⁾ However, some tumor cells, including malignant gliomas, secrete an active form of TGF- β .^{2,19,25)}

Our previous studies^{38,39)} showed that LAK cells secreted TNF- α , TNF- β , and interferon- γ , and the secretion increased when LAK cells were cultured with non-gliomatous tumor cells. Malignant glioma cells, however, failed to stimulate secretion of these cytokines. We also showed that TGF- β secreted by glioblastoma cells inhibited the secretion of TNF- α and TNF- β by LAK cells.⁴⁰⁾ Therefore, if malignant glioma cells secrete TGF- β and can activate latent TGF- β , the active form of TGF- β may suppress the cytokine secretion by activated lymphocytes *in vitro* or *in vivo*. Recent reports suggest TGF- β is a factor in immunosuppression in patients with malignant gliomas.^{26,33)} Therefore, it is important to investigate TGF- β secretion in malignant gliomas.

This study examined the secretion of TGF- β 1 and TGF- β 2 in one glioblastoma cell line and seven surgically resected malignant glioma cell lines using a specific bioassay.⁴¹⁾

Materials and Methods

I. Collection of conditioned medium

Sterile surgical specimens from three glioblastomas and four anaplastic astrocytomas were cut into small pieces with scissors and passed through a syringe with a 18-gauge needle several times and then cultured in RPMI1640 medium containing 10% fetal calf serum supplemented with 2 mM glutamine and 100 μ g/ml kanamycin in tissue culture flasks (75 cm²). The medium was replenished every 4 or 5 days. After 2–3 weeks, the tumor cells formed confluent monolayers that could be passaged. Subconfluent human glioblastoma cell line A172 (Japanese Cancer Research Resources Bank, Tokyo) and surgically resected tumor cells within four passages were washed three times with RPMI1640 medium without serum and cultured in 3 ml of RPMI1640 medium without serum in tissue culture flasks (25 cm²) at 37°C in 5% CO₂. After 48 hours, the supernatants were collected, filtered (0.22 μ m), and stored at –20°C until use.

II. Immunohistochemical characterization of surgically resected tumor cells

Surgically resected malignant glioma cells were cultured on slides (Nunc Inc., Naperville, Ill., U.S.A.) and fixed in 10% formalin in phosphate buffered saline (PBS) for 30 minutes. After PBS washes, the slides were pretreated with ethanol for 30 minutes at –20°C, 0.3% H₂O₂ in methanol for 1 hour, and normal goat serum for 16 hours. The slides were then stained for glial fibrillary acidic protein (GFAP) and S-100 protein using DAKO LSAB Kit (DAKO Corp., Carpinteria, Cal., U.S.A.). Non-immunized rabbit immunoglobulin was used as a control in the primary reaction.

III. Cytokines and antibodies

Recombinant human TGF- β 1 (King Jozo Co., Ltd., Hyogo) and TGF- β 2 (Genzyme Corp., Cambridge, Mass., U.S.A.) were dissolved in 0.5 mM HCl and stored at –80°C until use. Preliminary studies indicated that at least 10 μ g/ml of both anti-TGF- β 1 (R&D Systems, Minneapolis, Minn., U.S.A.) and anti-TGF- β 2 (R&D Systems) antibodies neutralized completely 2.5 ng/ml of recombinant TGF- β 1 and TGF- β 2, respectively, and that anti-TGF- β 1 antibody did not neutralize recombinant TGF- β 2 and vice versa. Recombinant TGF- β 1 and TGF- β 2, and antibodies were diluted in RPMI1640 medium containing 10% fetal calf serum prior to use.

IV. TGF- β bioassay using Mv1Lu cells

The levels of TGF- β 1 and TGF- β 2 in the culture supernatants were measured by modified procedures⁴¹⁾ of the growth inhibition assay described elsewhere.^{5,56)} The culture supernatants from A172 and surgically resected tumor cells were acid-activated (1.5 μ l 6 N HCl/200 μ l supernatant) for 15 minutes at room temperature and then neutralized with 1.5 N NaOH to a final pH of 7–7.4, or left untreated. To determine whether the growth inhibition of Mv1Lu cells (donated by Dr. H. Fujiwara, Biomedical Research Center, Osaka University Medical School, Osaka) is due to TGF- β 1 or TGF- β 2, the untreated and acid-treated supernatants were pretreated with either anti-TGF- β 1 or anti-TGF- β 2 antibody (20 μ g/ml) or both antibodies together for 1 hour at 37°C. Fifty-microliter aliquots of TGF- β 1 and TGF- β 2 (5 ng/ml), and the supernatants from A172 and surgically resected tumor cells were plated in 96-well plates in duplicate and serial two-fold dilutions made in the plates. Fifty-microliter aliquots of Mv1Lu cells (2500 cells) were added to each well. After 72 hours at 37°C in 5% CO₂, wells were decanted and cells stained with 0.5% crystal violet in 20% methanol for 5 minutes at room temperature. The dye was eluted with 50% ethanol, and the absorbance at 570 nm was determined by a Titertek Multiskan (Flow Laboratories Inc., Helsinki, Finland).

Control Mv1Lu cells for growth inhibition assays were cultured in medium without recombinant TGF- β 1 or TGF- β 2, and were defined as 100% viable. The

Fig. 1 Controls for the bioassay. Recombinant TGF- β 1 and TGF- β 2 were used as standards. ■: recombinant TGF- β 1 (5 ng/ml); □: recombinant TGF- β 2 (5 ng/ml).

sensitivity of target cell populations to the effects of recombinant TGF- β 1 or TGF- β 2 was expressed as the percentage of viable cells. By determining the dilution of the test samples at which 50% inhibition occurs and extrapolating from the known recombinant TGF- β 1 and TGF- β 2 dilution curve (Fig. 1), the concentrations of TGF- β 1 and TGF- β 2 in the test samples were calculated.

V. Effect of TGF- β on the growth of glioblastoma cells

To examine the effect of TGF- β on the growth of glioblastoma cells, two glioblastoma cell lines, A172 and T98G (Japanese Cancer Research Resources Bank), were cultured in the presence of TGF- β 1 or TGF- β 2. Serial two-fold dilutions of recombinant TGF- β 1 (5 ng/ml) or recombinant TGF- β 2 (5 ng/ml) were made in 96-well microtiter plates. A172 or T98G cells (1500 cells/well) were seeded in each well. Control wells did not contain TGF- β 1 or TGF- β 2. Mv1Lu cells which are sensitive to both TGF- β 1 and TGF- β 2 were used as a control cell line. After 4 days, wells were decanted and stained with crystal violet. The dye was eluted and the absorbance was determined as described previously.

Results

I. Immunohistochemical characterization of surgically resected tumor cells

Immunohistochemical examination demonstrated that most cells cultured from the surgical specimens (within 4 passages) were positive for both GFAP and S-100 protein (Table 1).

Table 1 Immunohistochemical characterization of surgically resected tumor cells

Sample No.	GFAP	S-100 protein
Glioblastoma:		
1	++	++
2	+	+
3	++	++
Anaplastic astrocytoma:		
4	++	++
5	+	+
6	+	++
7	++	+

+: positive, ++: intensely positive.

II. TGF- β content in the culture supernatants from malignant gliomas

The total TGF- β 1 and TGF- β 2 contents in the culture supernatant from A172 were measured by acid activation, and the activities were specified by neutralization with anti-TGF- β 1 and anti-TGF- β 2 antibodies (Fig. 2). The TGF- β activities of the untreated and acid-treated supernatants were neutralized almost completely with the combination of both antibodies. Therefore, the activity after neutralization with anti-TGF- β 1 antibody is due to TGF- β 2, and vice versa.

The concentrations of active and total TGF- β 1 were 0.92 and 2.3 ng/ml, respectively, and of TGF- β 2 were 0.11 and 1.2 ng/ml, respectively. Acid treatment and addition of antibodies did not affect the viability of Mv1Lu cells. Using the same methods, TGF- β 1 and TGF- β 2 activities in the culture super-

Fig. 2 TGF- β activity in untreated (left) and acid-activated (right) culture supernatants from A172 cells. ■: untreated (left) or acid-activated (right), without antibody; □: pretreated with anti-TGF- β 1 antibody (20 μ g/ml); ●: pretreated with anti-TGF- β 2 antibody (20 μ g/ml); ◐: pretreated with both anti-TGF- β 1 and anti-TGF- β 2 antibodies.

Table 2 TGF- β activity in the culture supernatants from A172 and surgically resected malignant glioma cells

Sample No.	TGF- β 1 (ng/ml)		TGF- β 2 (ng/ml)	
	Untreated	Acid treated	Untreated	Acid treated
Glioblastoma:				
A172	0.92 (41%)	2.3	0.11 (9.7%)	1.2
1	weak	1.9	0.13 (7.8%)	1.7
2	0.41 (34%)	1.2	0.07 (5.8%)	1.2
3	0.87 (44%)	2.0	0.43 (26%)	1.7
mean $\pm$ SD		1.9 $\pm$ 0.4		1.6 $\pm$ 0.8
Anaplastic astrocytoma:				
4	weak	2.7	weak	2.3
5	UD	0.68	UD	0.82
6	0.79 (40%)	2.0	weak	0.51
7	0.55 (45%)	1.2	0.39 (49%)	0.79
mean $\pm$ SD		1.5 $\pm$ 0.3		1.1 $\pm$ 0.7

TGF- β 1 or TGF- β 2 activities are expressed as the doses equivalent to recombinant TGF- β 1 or TGF- β 2 used in the assay, respectively. "Weak" means that there was some activity but the value was too small to be calculated. Values in parentheses show the percentages of active forms of TGF- β 1 or TGF- β 2 (untreated) in the total amounts of TGF- β 1 or TGF- β 2 (acid treated). UD: undetectable.

natants from surgically resected tumor cells were measured (Table 2). Both active and latent forms of TGF- β 1 and TGF- β 2 were detected in the culture supernatants, except in one patient with anaplastic astrocytoma. The total TGF- β 1 and TGF- β 2 levels in

the culture supernatants were 1.9–2.3 and 1.2–1.7 ng/ml, respectively, in glioblastomas, and 0.68–2.7 and 0.51–2.3 ng/ml, respectively, in anaplastic astrocytomas. The TGF- β activities of the culture supernatants were neutralized completely with the antibodies.

Fig. 3 Effect of recombinant TGF- β 1 and TGF- β 2 on the growth of glioblastoma cells. $\circ$: T98G with TGF- β 1; $\bullet$: T98G with TGF- β 2; $\triangle$: A172 with TGF- β 1; $\blacktriangle$: A172 with TGF- β 2; $\square$: Mv1Lu with TGF- β 1; $\blacksquare$: Mv1Lu with TGF- β 2.

III. Effect of TGF- β on the growth of glioblastoma cells

Neither recombinant TGF- β 1 nor recombinant TGF- β 2 affected the growth of A172 and T98G cells, while the growth of Mv1Lu cells was inhibited in a dose-dependent manner (Fig. 3).

Discussion

I. Secretion of TGF- β 1 and TGF- β 2 by malignant gliomas

Previous studies have shown that glioblastoma cells secrete only TGF- β 2,^{2,11,12,59} although the messenger ribonucleic acid for TGF- β 1 and TGF- β 2 is present in glial tumors.^{19,32,55} TGF- β secretion by malignant glioma cells has not been thoroughly examined, since a method of quantitative bioassay for TGF- β has not been available. In contrast to previous studies, our present study indicated that culture supernatants from glioblastoma and anaplastic astrocytoma cells contained both active and latent forms of TGF- β 1 and TGF- β 2, and the total TGF- β contents were similar for glioblastomas

and anaplastic astrocytomas. In most cells, TGF- β is secreted in the latent form.^{8,28,34,35} The mechanism of activation of latent TGF- β remains unclear, but may involve proteases, such as plasmin and cathepsin D,²⁹ or the removal of carbohydrates.³⁴ These mechanisms are physiological, while activation by acidification is not, as a pH of less than 4 is required.^{29,34} The presence of the active form of TGF- β in our *in vitro* study may result from some malignant gliomas having a mechanism for the physiological activation of a latent form of TGF- β not requiring plasmin, or secreting the active form of TGF- β .

TGF- β inhibits the proliferation of many cell lines, both neoplastic and non-neoplastic.^{46,52} Some malignant cells, however, may lose sensitivity to growth regulation by TGF- β ,^{52,53} probably due to a failure to synthesize or release TGF- β , loss of receptors for TGF- β , or a failure in the intracellular TGF- β signaling pathway. TGF- β receptors are subclassified into three types, type I (65 kd), type II (85–95 kd), and type III (250–350 kd).⁹ The presence of type-I TGF- β receptor is essential for growth inhibition by TGF- β .³ Loss of receptors for TGF- β has been shown in some malignant gliomas.¹⁹ In our study, malignant glioma cells continued to grow *in vitro* despite the presence of active TGF- β . Furthermore, an excess of recombinant TGF- β 1 or TGF- β 2 (5 ng/ml respectively) did not affect the growth of glioblastoma cells. This agrees with the hypothesis that the insensitivity of malignant glioma cells to growth inhibition by TGF- β may result from a failure in the intracellular signaling pathway or loss of receptors for TGF- β .

Several other biological functions of TGF- β may contribute to tumor cell growth. TGF- β indirectly enhances the formation of extracellular matrix^{18,47,53} by increasing the secretion of plasminogen activator inhibitor.²⁷ Plasminogen activator inhibitor, produced by glioblastoma cells,^{24,36,50} may inhibit the proteolytic degradation of extracellular matrix. Also, TGF- β induces angiogenesis *in vivo*,⁴⁷ and is a potent chemotactic agent for monocytes⁵⁷ which secrete TGF- β 1 and fibroblast growth factor.¹ Fibroblast growth factor is also a potent angiogenic factor *in vivo*.¹⁷ Thus, TGF- β may act directly and indirectly to induce the formation of extracellular matrix and angiogenesis, resulting in the formation of good conditions for the growth of glioma cells.

II. Clinical implications

Patients with malignant gliomas manifest immunosuppression of the humoral and cell-mediated immune system.^{6,7,31} Recent studies have demon-

strated that peripheral blood lymphocytes obtained from patients with primary intracranial tumors respond poorly to mitogens and/or antigens.^{4,49,58} The immunosuppressive factor produced by gliomas, called the glioblastoma-derived T cell suppressor factor,^{51,59} is identical with TGF- β 2.¹² TGF- β has a variety of immunosuppressive properties, which are mediated by active TGF- β . Our observations *in vitro* showed that some TGF- β secreted by malignant gliomas was present as the active form. *In vivo*, active TGF- β secreted and activated by malignant glioma cells may inhibit the local immune reaction to malignant glioma cells by inhibiting secretion of cytokines such as TNF- α and TNF- β from activated lymphocytes. This could enhance tumor growth by allowing escape from the immunosurveillance system. TGF- β also causes preferential suppression of the CD4+ helper T cell function.⁵⁴ These findings agree with recent reports^{26,33} which suggest that TGF- β is a factor in immunosuppression in patients with malignant glioma.

Patients with malignant gliomas have been treated with LAK cells,^{37,42,60} but the efficacy was limited. Our previous study suggested that, if tumor cells have the capacity to activate TGF- β , the active TGF- β suppresses the secretion of TNF- α and TNF- β by LAK cells stimulated by the tumor cells.⁴⁰ The present study showed that most malignant glioma cells had the capacity to activate TGF- β . This may reduce the efficacy of immunotherapy using LAK cells.

Our study showed that culture supernatants from malignant glioma cells contained both active and latent forms of TGF- β 1 and TGF- β 2, except in one case of anaplastic astrocytoma. The presence of the active form of TGF- β in the supernatants is an important finding. Malignant glioma cells may have a mechanism for the activation of latent TGF- β and may suppress cytokine secretion by immune effector cells *in vivo* as well as *in vitro*. This may inhibit the immune reaction against tumor cells and be a factor in the immunosuppressive state of patients with malignant glioma.

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