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Detection of Active Form of Transforming Growth Factor-β in Cerebrospinal Fluid of Patients with Glioma

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We examined transforming growth factor-β (TGF-β) activity in cerebrospinal fluid of 39 patients with various brain tumors, and found it in 10 glioma cases that had lesions related to subarachnoid space or ventricle. In one glioma case, TGF-β detected on admission disappeared after radiation and chemotherapy. We confirmed that five glioma cell lines produced TGF-β, and that four of them produced active form of TGF-β directly. The active form of TGF-β was also identified from cerebrospinal fluid before the acidification treatment in two cases. The calculated contents were 110 ng/ml and 18 ng/ml. These results indicate that active form of TGF-β is directly produced by tumor cells in patients with glioma, and may contribute to immunodeficiency of the host.

Key words: TGF-β — Cerebrospinal fluid — Glioma — Brain tumor

Transforming growth factor-β (TGF-β) is one of a large gene family of structurally related cell-regulatory proteins.[1] TGF-β exerts multiple actions on various cells including immune cells.[2] Recently, enhanced expression of TGF-β gene and excessive production of TGF-β in many malignant cell lines have been reported.[3] The plasma TGF-β levels were reported to be high in patients with hepatocellular carcinoma.[4] We have been investigating the $CD4^+$ helper T cell-selective suppression in the tumor-bearing state, and proved that TGF-β produced by tumor cells induced deleterious effects on T cells, especially on the $CD4^+$ helper T cell subset, in experimentally induced mouse tumor models.[5] In this study, we investigated TGF-β activity in cerebrospinal fluid of various brain tumors, and found TGF-β activity in some patients with glioma.

MATERIALS AND METHODS

Cells and reagents Mv1Lu cells and NRK49F cells for TGF-β assays were obtained from American Type Culture Collection (Rockville, MD). Human glioblastoma cell lines U251MG, U251SP and AJ were used. Two other glioma cell lines were established from our surgical specimens. FRKW was established from a surgical specimen of Case 10. AOYG was derived from a 35-year-old female with glioblastoma. Human recombinant (r) TGF-β1 and anti-TGF-β1 antibody were purchased from King Jozo Co. (Hyogo). Epidermal growth factor (EGF) was purchased from Sigma Chemical Co. (St. Louis, MO).

Cerebrospinal fluid and culture supernatant Cerebrospinal fluid was obtained from 39 patients with various brain tumors; 14 gliomas, 8 neurinomas, 6 germ cell tumors, 3 meningiomas, 2 craniopharyngiomas, 2 pituitary adenomas, 1 medulloblastoma, 1 metastatic brain tumor, 1 angioma, and 1 unknown tumor. The histological subtypes of the 14 gliomas are: glioblastoma, 5 cases; astrocytoma grade III, 4 cases; grade II, 1 case; ependymoma, 3 cases; ganglioglioma, 1 case. Glioma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 300 μg/ml L-glutamine. After the cells had grown to confluence in culture flasks (Falcon #3013), the medium was replaced with 10 ml of DMEM. The serum-free culture supernatants (SNs) were collected after 24 h.

Treatment of cerebrospinal fluid and culture supernatant with acid Cerebrospinal fluid and SNs were dialyzed against 1 *N* acetic acid and then against PBS, pH 7.4. This procedure is described as "A/N" treatment.

Detection of TGF-β activity

Mv1Lu cell assay The growth inhibition assay was performed according to the original method with slight modifications.[6] Briefly, Mv1Lu cells (1×10^4) were cultured with diluted samples or rTGF-β1 in 0.2 ml of DMEM containing 5% FBS in 96-well microplates for 24 h in a CO_2 incubator. Cells were pulse-labeled with 20 kBq of ^{3}H-TdR for the final 4 h, and the incorporated radioactivity was measured. Results are shown as the mean cpm ± SE of triplicate cultures.

NRK49F cell assay The procedure was essentially the same as previously described.[7] Agar plates were prepared in 60-mm petri dishes by first applying a 2-ml base layer of 0.5% agar in DMEM containing 10% FCS. Over this basal layer was added an additional 2-ml layer of 0.3% agar in DMEM/FCS containing an appropriate concentration of samples, 2×10^3 NRK49F cells and 2 ng/ml of EGF. Serially diluted rTGF-β1 at a concentra-

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tion from 0 to 100 ng/ml was used for the standard curve. These plates were placed in a CO_2 incubator (95% air/5% CO_2) for 10 days. Colonies were counted by using a microscope. The values indicate the mean number of colonies of duplicate cultures ± SE from individual experiments. The TGF-β content in the samples was determined from the standard curve by extrapolation of the values counted.

RESULTS

Secretion of TGF-β from glioma cell lines TGF-β activity in culture SNs was examined in terms of ^{3}H-thymidine uptake of Mv1Lu cells. Figure 1 shows the results for the crude and A/N treated culture SNs of five cell lines; FRKW, AOYG, U251MG, U251SP and AJ. Because of coincidental factors, the incorporation rate of ^{3}H-thymidine into Mv1Lu cell was variously affected, but a dose-response relation indicating that the TGF-β activity was due to rTGF-β1 was obtained from all A/N treated culture SNs and four crude SNs except for FRKW. DMEM was used as a negative control. This means that FRKW secretes only latent form of TGF-β, and all other cell lines secrete active TGF-β *in vitro*.

Detection of TGF-β in cerebrospinal fluid Even if cerebrospinal fluid is processed with acid, TGF-β activity cannot be detected cerebrospinal fluid under normal conditions (data not shown). We examined TGF-β activity in 39 cases of A/N treated cerebrospinal fluid of patients with various brain tumors with Mv1Lu cells. Twelve cases revealed TGF-β activity. Ten of them were gliomas. Others were an angioma and an untreated tumor. Figure 2 shows the results for all glioma cases in this study.

TGF-β activity was found in Cases 1 to 10, but not in Cases 11 to 14. In particular, the change of TGF-β activity before and after radiation and chemotherapy in Case 4 is noteworthy (Fig. 3): the TGF-β activity detected before therapy disappeared after the therapy.

Characteristics of gliomas in cases where cerebrospinal fluid contained TGF-β Table I shows the clinical data of 14 gliomas in this study. Each ependymoma grew in the subarachnoid space or the ventricle wall. A ganglioglioma grew at the lateral ventricle wall. Malignant astrocytomas and glioblastomas in Cases 1, 2, 4, 5, 7, 9 and

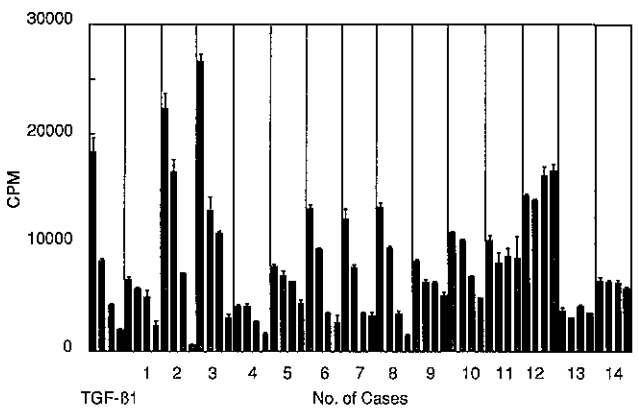

Fig. 2. TGF-β activity in A/N-treated cerebrospinal fluid of 14 gliomas in this study. Mv1Lu cells were cultured for 24 h in the presence of rTGF-β1 or A/N-treated cerebrospinal fluid of gliomas at 0.13%, 0.25%, 0.50% and 1.00% concentrations. The concentrations of rTGF-β1 used as the control are 0, 1.3, 2.5, 5 ng/ml. Values are means ± standard error of the means.

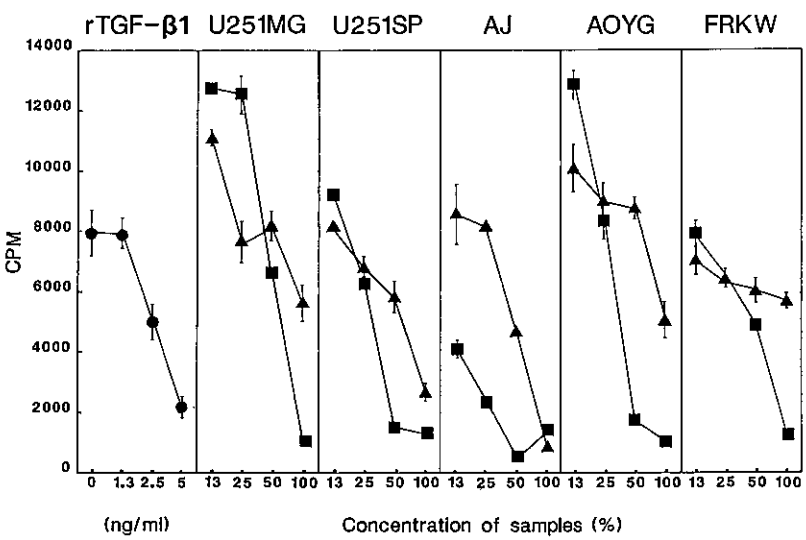

Fig. 1. TGF-β activity of one-day culture SNs of five glioma cell lines: U251MG, U251SP, AJ, AOYG, and FRKW. The TGF-β activities in crude (triangles) and A/N-treated (squares) culture SNs at various concentrations were measured with Mv1Lu cells. Human rTGF-β1 (circles) were used as the control. Values are means ± standard error of the means.

10 accompanied leptomeningeal invasions. On the contrary, glioma in Cases 10 to 14 were located in the brain parenchyma and had no lesions related to subarachnoid space or ventricle. These clinical findings suggest that the tumor cells in the subarachnoid space or ventricle secreted TGF-β into cerebrospinal fluid. FRKW was confirmed to secrete TGF-β *in vitro*, and the cerebrospinal fluid of the host (Case 10) was also confirmed to contain TGF-β.

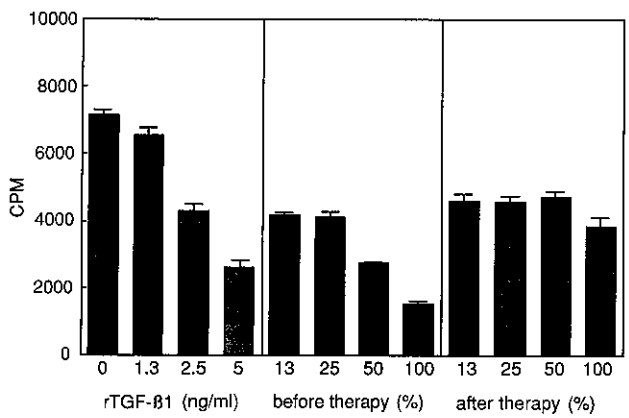

Fig. 3. Change of TGF-β activity in A/N-treated cerebrospinal fluid of Case 4 before and after therapy. Mv1Lu cells were cultured for 24 h in the presence of cerebrospinal fluid and rTGF-β1. Values are means$\pm$standard error of the means.

Detection of active form of TGF-β in cerebrospinal fluid

To detect active form of TGF-β in cerebrospinal fluid before A/N treatment, we adopted soft agar assay with NRK49F cells. Table II shows the results of four cases. EGF alone can induce 131$\pm$15 colonies without TGF-β. Active form of TGF-β can induce colony formation of NRK49F cells dose-dependently in the presence of EGF (2 ng/ml). The colony counts of Case 11 and Case 12 were at similar levels to the base line. On the other hand, the colony counts of Case 1 and Case 6 are 236$\pm$43 and 181$\pm$17, showing distinct colony-forming activity in the cerebrospinal fluid. The calculated TGF-β contents of crude cerebrospinal fluid of Case 1 and Case 6 were 110 ng/ml and 18 ng/ml, respectively.

DISCUSSION

TGF-β is a 25 kDa polypeptide, which was originally discovered as a factor to support the anchorage-independent growth of normal rat kidney cells.[8] The major subtypes are TGF-β1 and TGF-β2. TGF-β2 had been described as human glioblastoma-derived T cell suppressor factor.[9, 10] They are interchangeable in most biological assay systems.[6] As tumor cells might secrete both TGF-β1 and TGF-β2,[11, 12] we employed the two bioassay systems. In 39 cases of brain tumors, TGF-β activity was found in twelve cases. Ten of them were gliomas. Common clinical characteristics were the existence of lesions related to the ventricle or subarachnoid space. Especially in Case 4, the elevated TGF-β activity detected before the therapy was decreased by the radia-

Table I. Characteristics of Patients with Glioma in This Study

Case No.	Histological diagnosis[a]	Main lesion	Associated lesions	TGF-β in A/N cerebrospinal fluid[b]
1	GB	Temporal lobe	multiple seedings	+
2	GB	Cingulate gyrus	LI	+
3	EP	Frontal base	no	+
4	AS III	Temporal lobe	LI	+
5	GB	Parietal lobe	LI	+
6	EP	Vermis	multiple seedings	+
7	EP	Cervical spinal cord	syringomyelia	+
8	GG	Lateral ventricle	no	+
9	GB	Lateral ventricle	no	+
10[c]	GB	Parietal lobe	LI	+
11	AS III	Frontal lobe	no	−
12	AS III	Thalamus	no	−
13	AS III	Thalamus	no	−
14	AS II	Thalamus	no	−

a) Abbreviations: GB=glioblastoma; EP=ependymoma; AS=astrocytoma; III=grade 3; II=grade 2; LI=leptomeningeal invasion.
b) TGF-β activity was measured by using ^{3}H thymidine uptake of Mv1Lu cell proliferation.
c) FRKW cells were derived from surgical specimen of Case 10.

Table II. Existence of TGF-β Activity in Crude (before A/N Treatment) Cerebrospinal Fluid of Gliomas

No.	EGF (ng/ml)	Sample[a)]	Concentration	Colony counts[b)]
1	non	Medium only		0±0
2	2			131±15
3	2	rTGF-β1	100 ng/ml	302±18
4	2		10 ng/ml	237±7
5	2		1 ng/ml	161±22
6	2		0.1 ng/ml	136±27
7	non	Case 1 cerebrospinal fluid	10%	4±0
8	2		10%	236±43
9	non	Case 6 cerebrospinal fluid	10%	0±0
10	2		10%	181±17
12	non	Case 11 cerebrospinal fluid	10%	0±0
13	2		10%	107±3
14	non	Case 12 cerebrospinal fluid	10%	0±0
15	2		10%	117±6

a) rTGF-β1 or crude (before A/N treatment) cerebrospinal fluid of gliomas was added at the indicated concentrations to soft agar in anchorage-independent growth assay using NRK49F cells.
b) The values indicate the mean number of colonies of duplicate cultures±SE from individual experiments.

tion and chemotherapy. On the contrary, the cerebrospinal fluid of gliomas located in brain parenchyma did not contain TGF-β. These results indicate that glioma cells in subarachnoid space ventricle secreted TGF-β. It is reasonable that cerebrospinal fluid of glioma located in brain parenchyma, even if it produces TGF-β, does not contain TGF-β. The other two brain tumors were an angioma and an untreated tumor. The former was found after subarachnoid hemorrhage, TGF-β might have originated from platelets at the subarachnoid hemorrhage. The origin of TGF-β of the latter was unknown.

The unique feature of this study is the detection of TGF-β activity in not only A/N-treated but also crude (before A/N treatment) samples. TGF-β has been reported to be secreted in a latent form from various cells such as platelets, endothelial cells, macrophages and activated T cells, and converted to active form when needed.[12, 13] Some tumor cell lines have been reported to produce active TGF-β directly. We also confirmed that four glioma cell lines out of five secreted active TGF-β *in vitro*. However, even if the tumor cells secrete active form of TGF-β into blood vessels, such active TGF-β would be rendered inactive through binding to α_2-macrogloblin in serum.[14] On the other hand, the TGF-β released into cerebrospinal fluid would remain in active form because of the small content of α_2-macrogloblin and limited volume of cerebrospinal fluid in addition to the specific environment inside the blood brain barrier. Thus, this is the first report to describe the direct detection of the active form of TGF-β in cerebrospinal fluid of glioma patients, and the positivity was well correlated to the location of the tumors.

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