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DETECTION OF TNF INHIBITHORS (SOLUBLE RECEPTORS) IN THE SERA AND TUMOR CYST FLUID OF PATIENTS WITH MALIGNANT ASTROCYTOMAS OF THE BRAIN

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1. ABSTRACT

Patients with malignant astrocytomas of the brain exhibit varying degrees of immunosuppression with only a few factors responsible for this immunosuppression having been characterized. The soluble forms of the 55 kDa and 75 kDa membrane receptors for tumor necrosis factor (sTNF-R's) have been shown to bind to and inhibit the activity of TNF. The present studies analyze levels of sTNF-R's in the sera and tumor cyst fluids of patients with malignant astrocytomas. Using sensitive ELISA techniques, serum levels of the 55 and 75 kDa sTNF-R's in 17 patients tested were found to be elevated [55 kDa of 2.29 ± 2.85 ng/ml and 75 kDa of 4.98 ± 4.03 ng/ml] as compared to 20 normal controls [55 kDa of of 1.21 ± 0.91 ng/ml and 75 kDa of 1.85 ± 0.40 ng/ml] although this was only statistically significant for the 75 kDa sTNF-R (P=0.006). Brain tymor cyst fluid samples obtained from eight patients were shown to have very high levels of both sTNF-R's ranging from 4.16 to 17.17 ng/ml for the 55 kDa receptor and 4.83 to 19.96 ng/ml for the 75 kDa receptor. Six of these cyst fluid samples were also tested for their ability to inhibit TNF cytolytic activity using an *in vitro* assay. All samples tested had TNF inhibitory activity. Immunohistochemical studies on patient tumor samples showed high levels of expression of these receptors both in the cytoplasm and the cell surface of astrocytoma cells. We propose that sTNF-R's may be shed by astrocytoma cells and may have a role in both local and systemic immunosuppression observed in astrocytoma patients. Finally, the potential role of serum level of sTNF-R's as tumor markers to follow the treatment and the progression of disease in these patients are discussed.

2. INTRODUCTION

It is well established that patients with primary and recurrent malignant astrocytomas of the brain express varying degrees of immunosuppression. This is evidenced *in vivo* by reduced delayed hypersensitivity skin reactivity to different recall antigens and *in vitro* by reduced lymphocyte reactivity to various stimuli (1-3). Lymphocytes from these patients exhibit reduced proliferation *in vitro* when co-cultured with a variety of antigens and lectins as well as in mixed lymphocyte cultures (4-6). Furthermore, the *in vitro* response of normal lymphocytes to mitogens or IL-2 is suppressed when they are co-cultured with serum or tumor cyst fluid from glioblastoma multiforme patients or in mixed lymphocyte-tumor cultures (6-8). Moreover substances which inhibit lymphocyte proliferation *in vitro* are released by human glioblastoma cell lines *in vitro* (8-12).

Table 1. Clinical characteristics of patients from whom samples were tested

Patient	Age	Sex	Tumor Grade	Karnofsky Score
1	44	M	4	60
2	62	M	4	80
3	69	F	4	60
4	44	F	3	80
5	66	F	3	50
6	67	M	3	80
7	53	F	4	70
8	40	M	4	70
9	45	F	4	60
10	61	M	4	70
11	24	F	4	80
12	56	M	4	70
13	48	F	4	80
14	51	F	4	70
15	74	M	4	70
16	66	M	4	70
17	56	M	3	70
18	77	F	4	70
19	60	M	4	60
20	58	M	4	80
21	32	F	2/4*	80
22	32	F	3	70
23	42	F	4	20

* Pt. had grade II astrocytoma at the time tumor cyst fluid was taken but later progressed to grade IV.

In general, only a few factors(s) causing these inhibitory effects have been characterized including: Transforming growth factor-beta (TGF-β), Interleukin-10, Interleukin-6, and Prostaglandin E_2 (PGE_2) (8-13).

Recently, soluble cytokine receptors acting as cytokine inhibitors have been identified in cancer patients. These soluble cytokine inhibitors include IL-1 receptor antagonist (14), soluble IL-2 receptor (15) and the extracellular fragments of the 55 kDa and 75 kDa membrane receptors for tumor necrosis factor (sTNF-R's). In particular, sTNF-R's are found at high levels in the serum, ascites fluid and pleural effusions of patients with various types of malignancies (16-21) but there has been no reported study demonstrating their presence in patients with intracranial astrocytoma. These sTNF-R's form complexes with tumor necrosis factor-α (TNF) and lymphotoxin (LT, also known as tumor necrosis factor-β) and stearically inhibit the biological activity of these cytokines by competing with the specific membrane receptors on cells and tissues (22-23). While the cell source and role of sTNF-R's in cancer patients are still under investigation, it is clear that these molecules can inactivate TNF and LT biological effects *in vitro* and *in vivo* (24-25). The present studies were conducted to determine if sTNF-R's are present in the sera and tumor cyst fluid of patients with malignant astrocytomas.

3. MATERIALS AND METHODS

3.1. Clinical Characteristics of Patients

As shown in Table 1, 22/23 patients studied had anaplastic astrocytomas or glioblastoma multiforme (grade III or IV astrocytomas according to the Daumas-Duport classification (26)). One patient had an initial presentation with a grade II astrocytoma but later progressed to a grade IV astrocytoma (glioblastoma multiforme); tumor cyst fluid was taken from this patient at the time of the initially lower grade tumor. All patients had received traditional therapies including surgery, radiation and/or chemotherapy. No patient had received chemotherapy within two months prior to sample collection. The age ranged between 24 to 77 years with a mean age of 53 years. There were 11 males and 12 females. Karnofsky scores ranged from 20 to 80 with an average of 68. A total of 20 normal blood donors were randomly selected with equal numbers of males and females and ranged in age from 20 to 82 years (mean = 42.3 years). 20 normal healthy volunteers (10 males, 10 females) without previous history of cancer were used as controls.

3.2. Serum Collection

Peripheral blood was collected from normal individuals or patients with recurrent mallignant astrocytomas by venipuncture in a serum separator tube and was allowed to coagulate in the refrigerator for 24 hours at 4 C. Blood was collected from patients just prior to surgery. Cells and particulate debris were removed by centrifugation at 850 X G for 10 minutes and serum samples were tested immediately or frozen at –20 C until use. Pilot studies conducted with samples of patient and normal donor serum spiked with known levels of recombinant human TNF, and the 55 kDa and 75 kDa sTNF-R revealed that the immunologic and biologic activity of these molecules was not altered during these procedures.

3.3. Tumor Cyst Fluid Collection

Brain tumor cyst fluid was obtained either at the time of surgery for tumor resection or percutaneously via an Ommaya reservoir placed in the cyst cavity. Cells and particulate debris were removed by configuration at 850 x G for 10 minutes. Cell-free cyst fluid was then aliquotted and frozen at –20 C until the time of testing.

3.4. ELISA for TNF and sTNF-R's

Concentrations of TNF and sTNF-R's were determined by ELISA, employing our own polyclonal rabbit antibody against recombinant human TNF or the 55 kDa or 75 kDa sTNF-R. The specifications of these antibodies and the ELISA assays have been described previously (20). Recombinant human 55 and 75 kDa TNF-R proteins used to generate antibodies were kindly supplied by Synergen, Inc., (Boulder,CO). Recombinant human TNF was kindly supplied by Genentech (South San Francisco, CA). All samples were assayed in duplicate wells along with known standards, and color intensity was determined by spectrophotometric determination of optical density at the appropriate wavelength using an EAR 400 AT ELISA plate reader (SLT Lab Instruments, Salzburg, Austria). Standard linear regression lines were generated by plotting $\log_{10}$ concentration vs. $\log_{10}$ optical density. The amount of TNF, and each sTNF-R was determined by comparing the sample to the standard curve for the cytokine and each of the soluble receptors, respectively.

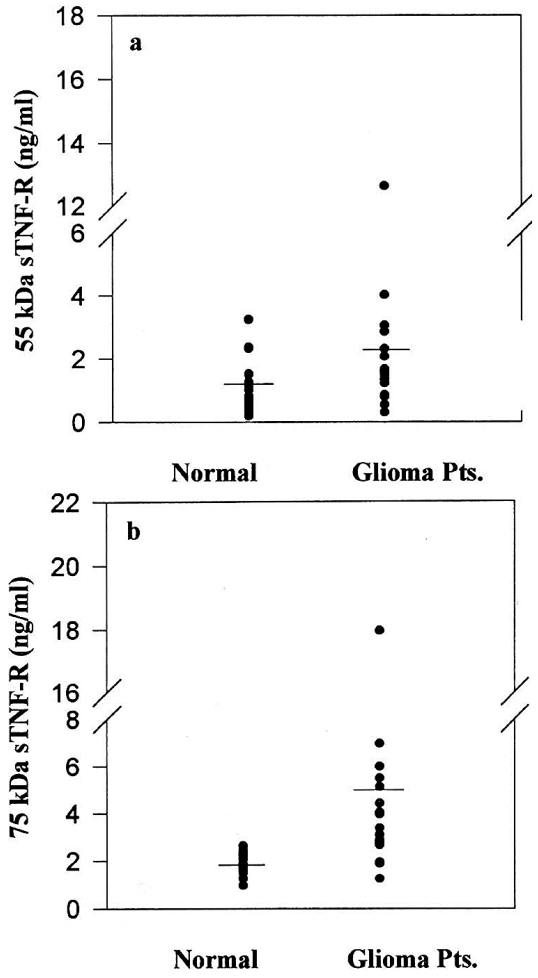

Figure 1. Serum levels of the 55 and 75 kDa sTNF-R's expressed in ng/ml in normal individuals and patients with high grade astrocytomas. (a) Comparison of the mean serum levels of the 55 kDa sTNF-R between normal individuals [1.21 ± 0.91 ng/ml] and patients with high grade astrocytomas [2.29 ± 2.85 ng/ml (P = 0.153)]. (b) Comparison of the mean serum levels of the 75 kDa sTNF-R between normal individuals [1.85 ± 0.40 ng/ml] and patients with high grade astrocytomas [4.98 ± 4.03 ng/ml (P =0.006)].

3.5. Cytolytic Bioassay for TNF Activity and TNF Blocking Factor Activity

TNF activity of brain tumor cyst fluids, and serum samples was assayed on L929 mouse fibroblasts as described (27). Brain tumor cyst fluids were then assayed for blocking of TNF cytolytic activity by bioassay using the L929 mouse fibroblasts as previously described (24). Briefly, serial dilutions of known amounts of recombinant human TNF were added to a constant amount of sample containing blocking factor activity or to control medium. After co-incubation at 37 C for 1 hour, 75ul of the mixture and 25ul of Actino-mycin D (8ug/ml) were added to duplicate wells of L929 cells in the cytolytic bioassay. After 16 to 24 hours of incubation at 37 C in 5% CO2/95% air, the media were aspirated and cells were stained with 1% crystal violet for 5 minutes, washed with water, and solubilized with 100ul of 100 mM HCL in methanol. The viable cell number was determined by the optical density at 590nm measured in an EAR AT ELISA plate reader. Curves plotting percent cell killing vs TNF concentration for the standard as well as the sample + TNF mixture were generated. An LD50, or the amount of TNF mixed into a given sample causing 50% cell lysis, was then determined for the TNF standard as well as for each of the samples.

3.6. Immunohistochemical Staining of Astrocytoma Tissue Sample

Tumor tissue removed at surgery was fixed in neutral buffered formalin. Sections were prepared on siliconized glass slides and stained using a Techmate automated immunostaining system (Biotech Solutions, Inc., Santa Barbara, CA) with the avidin-biotin complex method using DAB as the chromogen. Anti-p55 and anti-p75 TNF-receptor antibody (1:100 dilution based upon titration studies) were used as the primary antibodies and goat anti-rabbit IgG was used as the secondary antibody. After immunostaining, tissues were counter-stained with hematoxylin and examined under the microscope.

3.7. Statistical Analysis

Data were analyzed using the Student's *t* test with a P value <0.05 being considered significant. Where indicated, data were also analyzed using the correlation coefficient.

4. RESULTS

4.1. Serum Levels of TNF and the 55 kDa and 75 kDa sTNF-R's in Malignant Astrocytoma Patients and Normal Individuals

Serum samples were collected from 20 normal individuals and from 17 patients with recurrent high-grade astrocytomas. All samples were tested for levels of TNF and each sTNF-R by ELISA as described in Materials and Methods. The sensitivity of the ELISA assay for TNF was 0.02 ng/ml. While only 3/20 normal individuals (15%) had detectable levels of TNG (range = 0.07 to 0.88 ng/ml, mean 0.41 ± 0.42 ng/ml), 11/17 astrocytoma patients (65%) had detectable levels of TNF (range = 0.04 to 14.09 ng/ml, mean = 1.59 ± 4.15 ng/ml). However, of the samples from normal individuals and brain tumor patients with immunologically detectable TNF, none had any TNF cytolytic activity as determined by the L929 bioassay. Thus this TNF was inactive.

For normal individuals, the average values for the 55 and 75 kDa sTNF-R were 1.21± 0.91 ng/ml and 1.85 ± 0.40 njg/ml, respectively, and the levels were not significantly different between sexes. As shown in Figure 1a, when compared to normal individuals, mean serum levels for the 55 kDa sTNF-R in the astrocytoma patients were 2.29 ± 2.85 ng/ml. Although these levels were higher than normal controls, this did not reach statistical significance (P = 0.153). As shown in Figure 1b, mean serum levels for the 75 kDa sTNF-R in astrocytoma patients were 4.98 ± 4.03 ng/ml which was significantly higher than that of normal individuals (P = 0.006).

Table 2. Brain tumor cyst fluid samples of astrocytoma patients

Patient	TNF (ng/ml)	Bioactive TNF (ng/ml)	55 kDa sTNF-R (ng/ml)	75 kDa sTNF-R (ng/ml)	LD50 TNF Inhibition Assay* (ng/ml)
2	0.74	n.t.	10.63	19.96	n.t.
4	0.18	n.t.	4.58	9.61	n.t.
18	0.19	0	12.17	18.00	0.14
19	0.46	0	4.16	7.32	0.08
20	<0.02	0	4.75	4.62	0.11
21	<0.02	0	5.26	4.83	0.10
22	0.16	0	12.75	16.61	0.16
23	<0.02	0	17.17	10.91	>1.60

* LD50 for the TNF standard was 0.05 ng/mln.t. = not tested

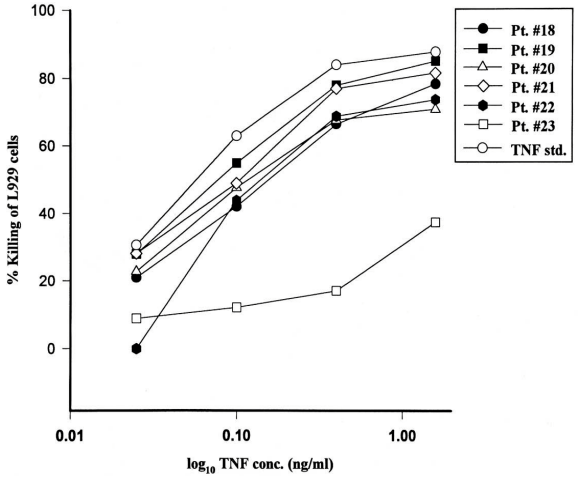

Figure 2. Graph illustrating blocking activity against human TNF-induced cytolysis of murine L929 cells *in vitro* by brain tumor cyst fluid samples. Serial dilutions of known amounts of recombinant human TNF were added to a constant amount of sample or to control medium. The sample + TNF mixtures or the TNF standard (-0-) were then incubated with murine L929 cells for the 16 – 24 hours and % killing of cells was obtained. All brain tumor cyst fluid samples had inhibitory activity against TNF.

4.2. TNF Levels in Brain Tumor Cyst Fluid Samples

Brain tumor cyst fluid was collected from 8 patients either at the time of surgery or percutaneously via an Ommaya reservoir placed into the cyst cavity. 5/8 samples had detectable levels of TNF as determined by ELISA (mean = 0.34 ± 0.25 ng/ml). However, of 6 samples that were tested for TNF activity, none had detectable levels of cytolytic activity using the L929 mouse fibroblast bioassay (Table 2). This material was also biologically inactive.

4.3. Levels of the 55 and 75 kDa sTNF-R's, and TNF Blocking Factor Activity in the Tumor Cyst Fluid From Patients With Malignant Astrocytomas

Using ELISA techniques, high levels of sTNF-R's were found in tumor cyst fluid samples. Mean values for the 55 kDa sTNF-R were 8.93 ± 4.91 ng/ml (range 4.16 – 17.17 ng/ml) and for the 75 kDa sTNF-R were 11.48 ± 6.01 ng/ml (rabge 4.62 – 19.96 ng/ml) (Table 2). Using the TNF blocking factor assay, 6 of the 8 cyst fluid samples were tested for TNF inhibitory activity. The LD50 for the TNF standard and for each sample + TNF mixture are listed in Table 2. All samples had higher LD50 levels than the TNF standard indicating the presence of TNF blocking factor activity in the cyst fluids. In general, the most inhibitory tumor cyst fluids were those that contained the highest levels of sTNF-R's. Specifically, there was a correlation between the level of TNF inhibition and the amount of the 55 kDa sTNF-R (r=0.74.). Figure 2 illustrates the % lysis of L929 cells vs. concentration of TNF for several patient samples (mixed with known concentrations of TNF) and for the TNF standard. As demonstrated, all the cyst fluids had TNF inhibitory acitivity as compared to the control medium.

4.4. Immunohistochemical Staining of Astrocytoma Tissue

Tumor tissue removed from patient #21 diagnosed as grade IV astrocytoma (glioblastoma multiforme) was analyzed for TNF-R expression using standard immuno-histochemical analysis. As can be seen in Figure 3, glioma cells strongly express both 55 kDa and 75 kDa TNF-R's. Strong membrane (as well as cytoplasmic) staining was noted for the 55 kDa TNF-R (Figure 3A) and strong cytoplasmic (with less obvious membrane) staining was noted for the 75 kDa TNF-R (Figure 3B). Negative controls (normal rabbit IgG) did not stain these tumor cells (Figure 3C). In contrast, cells in areas of normal brain tissue in the sample did not express detectable levels of either TNF receptors (not shown).

5. DISCUSSION

Previous studies have shown immunosuppressive substances in the sera, CSF, tumor cyst fluid and glioblastoma culture supernatants from patients with high grade astrocytomas (2;8-12;28-29).

Few of the factors responsible for these effects have been characterized. It is known that TGF-β and PGE_2 are produced by astrocytoma cells and both of these substances are capable of suppressing T-cell proliferation and function(13;30-33). TGF-β has also been shown to be present in the glioblastoma residual cavity after subtotal tumor resection (34). However, Couldwell, *et al*., reported that TGF-β and PGE_2 levels in supernatants from primary astrocytoma cultures did not correlate with levels of functional immunosuppression as tested by *in vitro* lymphocyte proliferation and suggested that other factors must be involved (35).

It has become apparent that the biologic activity of cytokines including IL-1, IL-2, IL-4, TNF and LT is regulated by release of soluble receptors or receptor antagonist. Soluble forms of TNF receptors compete with cell surface receptors for TNF and subsequently inhibit cytokine activity (22-23). TNF has important roles in the inductive and tissue destructive effector phases of cell-mediated immunity. In addition TNF has been shown to have multiple roles in malignancy, including direct cytotoxic and cytostatic effect on glioma cells, induction of IL-1 production, migration of lymphocytes and other

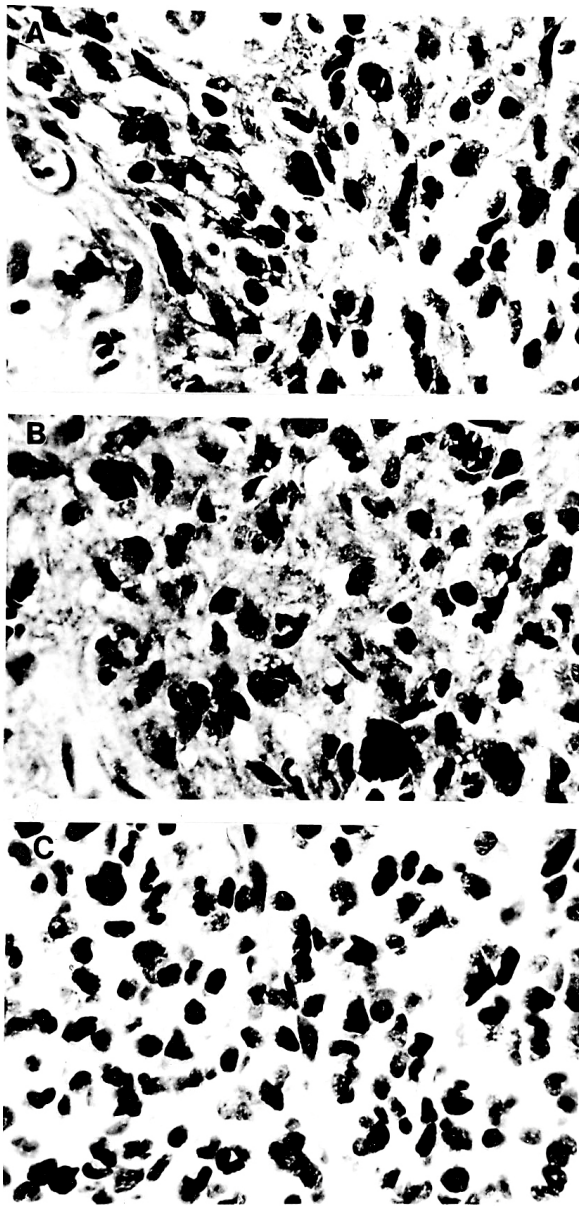

Figure 3. Immunostaining of 55 kDa and 75 kDa TNF-R on human glioblastoma cells. A) 55kDa TNF-R; B) 75 kDa TNF-r; C) Normal rabbit serum. Magnification 400x.

immune cells, upregulation of MHC and ICAM surface antigens on tumor cells and activation of polymorphonuclear neutrophils (36-39).

Gatanaga, *et al*., showed that sTNF-R's inhibited the necrotizing activity of recombinant human TNF in established cutaneous murine tumors (24). In another study, IL-2 induced lymphocytic infiltration of multiple organs in mice was significantly suppressed by the intraperitoneal administration of sTNF-R's (40). Since low levels of sTNF-R's are also present in the serum of normal individuals, it is possible that they reprent a baseline protection against serum released TNF. However, high levels of these receptors in the area of a tumor may facilitate a microenvironment that ultimately blocks the activity of TNF produced by infiltrating macrophages and lymphocytes. Thus, high levels of the sTNF-R's together with other inhibitors may result in local immunosuppression causing the tumor to escape the induction and the effector phases of cell-mediated immunity. Furthermore, chronically elevated levels of sTNF-R's in the serum may contribute to systemic immunosuppression seen in many cancer patients.

In this study, we analyzed serum and tumor cyst fluid levels of sTNF-R's in patients with malignant astrocytomas of the brain and we found elevated levels of both sTNF'R's in the sera of patients as compared to normal controls although this was only statistically significant for the 75 kDa sTNF-R. Our studies also revealed the presence of sTN-R's in the tumor cyst fluids of astrocytoma patients which were several-fold higher than the levels present in the serum. While the tumor cyst fluid samples also had variable levels of immunologically detectable TNF, none of the samples tested had any biologically active TNF and, in fact, all had TNF inhibitory activity. Thus, the presence of immuno-reactive but not bioactive TNF indicates that these molecules may be complexed with sTNF-R's. This was suggested by the fact that serum levels of TNF (in patients with detectable TNF levels) correlated very well with the serum levels of the 55 kDa sTNR-R ($r = 0.94$). However, it is interesting to note that this correlation did not exist with the serum levels of the 75 kDa sTNF-R. In general, tumor cyst fluids with the highest TNF inhibitory activity also had the highest amount of sTNF-R's and more specifically, the level of TNF inhibition correlated with the level of the 55 kDa sTNF-R ($r = 0.74$).

Immuno-histochemical staining revealed that astrocytoma cells expressed both receptors in the cytoplasm and on the cell membrane suggesting that these cells may be at least one possible source of sTNF-R's in the tumor microenvironment.

Using ELISA techniques, sTNF-R's have been shown to be elevated in the sera of patients with various other malignancies including ovarian, colon and endometrial carcinomas as well as hematological malignancies(16-20;38;41-43). In the present study, the 75 kDa sTNF-R was the predominant form in the sera of these astrocytoma patients. It has been shown that this is also the predominant form in the sera of patients with colon cancer and that levels of the 75 kDa receptor correlated with the stage of the disease.1 Several studies have suggested that the serum levels of sTNF-R's in patients with ovarian cancer may be more sensitive markers of active disease, stage of disease, and clinical response to therapy than the well-established serum CA-125 marker (17-19). In the present study, there was no difference in serum levels of these receptors between patients with grade III and those with grade IV astrocytomas. However, patients with low grade astrocytomas were not tested and a potential difference in sTNF-R's between low and high grade astrocytoma patients, if present, may be a valuable

diagnostic tool. To date, there is no widespread serum "tumor marker" that can be used to follow the course of astrocytoma patients. Radiological studies can often be confusing especially after surgical and radiation therapies and clinical symptomatology alone can preclude early diagnosis of recurrence or progression of disease. Thus, while serum levels of sTNF-R's are not tumor specific, they may play a role as "tumor markers" in following the course of patients with either a known or presumptive diagnosis of primary intracranial astrocytoma.

Mechanisms explaining the shedding of sTNF-R's and the cell source of origin are unclear. Our laboratory has demonstrated release of sTNF-R's from primary cell cultures of tumor tissue derived from glioblastoma patients (unpublished data). Our present study supports the fact that astrocytoma cells may be major source of these soluble receptors. The fact that tumor cyst fluid levels of sTNF-R's were 5 to 10 times higher than mean serum levels in astrocytoma patients indicates a local (as opposed to systemic) production in the area of the tumor. Moreover, immunohistochemical studies show that astrocytoma cells do express both forms of TNF-R's on the cell surface and in the cytoplasm. Several studies have demonstrated that tumor cells have an increased ability to shed soluble forms of cell surface proteins (44). It is possible that sTNF-R's may be produced and shed by tumor cells and such soluble receptors may circumvent or blunt immunological attack. If this is so, countering the effect of these molecules may be an important consideration in the development of new treatment strategies in immunotherapy of brain tumors. Finally, the clinical utility of monitoring serum levels of sTNF-R's in following progression of tumor, recurrence and response to therapy in these patients remains to be determined.

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