Effects of recombinant human tumor necrosis factor on rodent gliomas and normal brain

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In a study examining the possible therapeutic effects of recombinant human tumor necrosis factor-alpha (rHuTNF-α) on malignant gliomas without expression of tumor necrosis factor (TNF)-receptors, RG-2 glioma cells were tested in vitro as well as in a rat experimental glioma model. A growth inhibition assay revealed no inhibiting effect in vitro up to a concentration of 20 μg/ml rHuTNF-α. Receptor-binding studies showed that RG-2 cells did not present specific receptors for rHuTNF-α. The pharmacokinetics of rHuTNF-α after intravenous injection were studied with respect to serum, tissue, and brain tumor concentrations and showed increased glioma concentrations of (mean ± standard error of the mean) 0.47 ± 0.18 ng TNF/mg brain compared to 0.15 ± 0.05 ng TNF/mg brain in the normal contralateral hemisphere.

No therapeutic effect on solid RG-2 gliomas could be observed after stereotactic injection of 7.3 μg rHuTNF/10 μl buffer solution into the tumor in 10 animals. Immunohistochemical studies after stereotactic injection of rHuTNF-α showed total disappearance of the substance after 24 hours without internalization into tumor cells. Stereotactic injection of 7.3 μg rHuTNF10 μl into normal brain resulted in marked inflammatory response around the injection track, including microvascular thrombosis. These results demonstrate that rHuTNF has neither direct nor indirect cytotoxic activity on RG-2 glioma cells. Furthermore, before clinical use of rHuTNF-α in malignant gliomas, the authors suggest that receptor studies be done in each patient. In receptor-positive patients undergoing treatment with rHuTNF-α, precautions should be taken to prevent local encephalitic reactions.

KEY WORDS: brain tumor • glioma • stereotaxy • tumor necrosis factor • immunotherapy • rat

Tumor necrosis factor (TNF), a protein of about 17 kDa as a monomer, is secreted by activated monocytes and belongs to a group of immunomediators that are commonly referred to as "biological response modifiers." Several investigators have reported its potent antitumoral activity against various murine and human tumors in vitro and against primary cultures of malignant human brain tumors. Systemic application of TNF-containing serum in 9L gliosarcoma-bearing rats resulted in prolonging the survival time of animals in this experimental sarcoma model. Furthermore, there is evidence that TNF not only possesses direct cytotoxic capacities against tumor cells with TNF-specific surface binding sites but also shows indirect immunologically dependent antitumor activities.

In contrast, other authors have shown a growth-promoting effect of TNF on the U373 glioma line in vitro. Furthermore, investigators could not show beneficial therapeutic effects of the substance in tumor xenografs (astrocytoma, oligodendroglioma, leiomysarcoma) of the nude mouse. Preliminary clinical trials with recombinant human TNF-alpha (rHuTNF-α) have been conducted in patients with advanced cancer, but the overall antitumor effects were unsatisfactory. Various systemic side effects were observed after application of rHuTNF-α, including fever, chills, hypotension, fatigue, anorexia, and headaches, but were mild and reversible.

Faced with these conflicting results, we decided to examine the biological effects of rHuTNF-α in the rat experimental RG-2 glioma model compared to normal brain tissue. Initially, we conducted in vitro studies to examine the receptor status of RG-2 cells in relation to rHuTNF-α and to screen for direct cytotoxicity. In subsequent studies, the pharmacokinetics of the sub-
stance were analyzed in vivo. Further studies investigated the possible therapeutic indirect (immunomodulatory/inflammatory-mediated) antitumor effects in vivo by means of stereotactic application of rHuTNF-α into intracerebral RG-2 gliomas to address the question of whether rHuTNF-α can exert therapeutic effects in vivo even in the absence of specific surface binding sites. Finally, stereotactic injections of the substance into normal brain were performed to examine the safety of this form of therapeutic drug-immunomediator delivery.

Materials and Methods

All experiments were performed with CD Fischer 344 rats and were conducted at the Central Institute for Animal Research of the Heinrich-Heine-University, Düsseldorf, Germany. The experiments were approved by the Government of North Rhine-Westphalia, represented by the Regierungspresident, Düsseldorf, and were performed strictly in accordance with federal law. We used RG-2 glioma cells, a cell line that was originally described by Ko, et al., and has since been investigated extensively by several authors. For the in vivo experiments, solid gliomas were induced by stereotactic injection of 10⁶ RG-2 cells suspended in 10 μl Dulbecco's minimum essential medium (MEM) into the right caudate nucleus of rats, using a stereotactic frame. The experiments were carried out 7 days after the tumor cell injection, when the tumors had a median diameter of about 3.5 mm on frontal sections. Histological or functional evaluation by radioisotope scanning was performed on Day 14, after injection of tumor cells.

Receptor Binding Assay

The expression of receptors in RG-2 cells for rHuTNF-α was studied. The RG-2 cells were maintained in MEM culture supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, nonessential amino acids (1:200 dilution), and 50 μg/ml gentamicin. All experiments were performed at the exponential cell growth phase. The specific activity of rHuTNF-α was approximately 8 x 10⁵ U/mg, determined by an actinomycin D (free cytotoxicity assay on murine L-929 cells. The lactoperoxidase method of Bolton and Lassiter was used to label rHuTNF-α with Na¹²⁵I, resulting in a final specific radioactivity of about 40 μCi/μg. The biological activity of iodinated rHuTNF-α was about 65% that of unlabeled rHuTNF-α. Binding assays of ¹²⁵I-rHuTNF-α were performed with 2 x 10⁴ RG-2 cells per group. For control studies, U937 cells (human histiocytic cell line with known expression of TNF receptors) were used. Cells were incubated at 0°C for 1 hour with increasing amounts (0.6 to 6 ng) of ¹²⁵I-rHuTNF-α in 0.3 ml phosphate-buffered saline containing 0.02% NaN₃ and 2% heat-inactivated FCS. All measurements were made in duplicate and corrected for nonspecific binding of ¹²⁵I-rHuTNF-α as measured with a 200-fold excess of unlabeled rHuTNF-α. After the cells were washed twice, the cell-bound radioactivity was determined and the number of receptors was calculated from the Scatchard analysis of the saturation binding data by linear regression.

Growth Inhibition Assay

The sensitivity of RG-2 glioma cells for rHuTNF-α was measured by a cell growth inhibition assay. For this, 100 μl of culture medium containing 2 x 10⁴ RG-2 cells was added to the wells of a microtiter plate. Then 100 μl of culture medium containing increasing concentrations of rHuTNF-α up to 20 μg/ml were added (six replicates) and incubated for 48 hours at 37°C in humidified air with 5% CO₂. During the last 4 hours of culture, the cells were pulsed with 1 μCi of tritiated thymidine (³H-TdR)/25 μl/well. The cells were collected with a semiautomatic cell harvester and radioactivity incorporated into deoxyribonucleic acid (DNA) was counted in a liquid scintillation counter. For control studies, U937 cells were used.

Pharmacokinetics of rHuTNF-α in RG-2 Glioma-Bearing Rats

Seven tumor-bearing rats were examined on Day 7 after stereotactic injection of 10⁴ RG-2 glioma cells. A 1-mg/kg dose of ¹²⁵I-rHuTNF-α was injected via the tail vein. In three animals, serum levels of ¹²⁵I-TNF were measured 2, 5, and 15 minutes after this injection. In these three animals, the ¹²⁵I-rHuTNF-α concentration was also measured in the liver, kidneys, heart, and lung after sacrifice and tissue homogenization. All sever-
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![Graph](image)

**Fig. 2.** Receptor binding assay for RG-2 cells, including the nonspecific binding (NSB). There is little difference between the curves, indicating a total lack of tumor necrosis factor (TNF) receptors.

![Graph](image)

**Fig. 3.** Serum concentrations of $^{125}$I-rHuTNF-α at 2, 5, and 15 minutes after intravenous injection of 1 mg/kg of the substance in three animals.

en animals were sacrificed 15 minutes after the intravenous injection. In six animals, the tumor area was excised then homogenized and measured by scintillation counter. For comparison, the surrounding hemisphere including the brain adjacent to the tumor area was measured in the same way. In three animals the contralateral, nontumor-bearing hemisphere was analyzed in the same manner to exclude the influence of the disrupted blood-brain barrier on the pharmacokinetic data.

**Stereotactic Treatment of RG-2 Glioma-Bearing Rats With rHuTNF-α**

Tumors were induced in 15 animals as described above. These tumor-bearing rats were treated stereotactically 7 days after injection of tumor cells. In 10 animals 7.3 μg rHuTNF-α dissolved in 10 μl of 50 mM Na-inorganic phosphate (P) buffer (pH 8.5) was given in a single injection over 1 minute. The other five animals were stereotactically injected with 10 μl of heat-inactivated human serum. The treatment effects were evaluated histologically on Day 14.

**Sequential Immunohistochemical Examination of rHuTNF-α**

To analyze the time course of rHuTNF-α after stereotactic injection into RG-2 gliomas, we conducted an immunohistochemical study as follows. Tumors were induced as described above in 19 rats. Again, 7.3 μg rHuTNF-α dissolved in 10 μl of 50 mM Na-P buffer (pH 8.5) was given in a single injection over 1 minute to each of 13 animals. In the other six animals, 10 μl inactivated human serum was injected into the tumor. The animals were sacrificed 5 minutes, 1 hour, 2 hours, 1 day, and 4 days after the injection. The brains were immediately prepared and frozen in liquid nitrogen. Immunohistochemical studies with monoclonal antibodies (anti-TNF-α, clone 2H-4) were performed on cryostat-cut sections. We used the indirect avidin-biotin-peroxidase (ABC) method, the second antibody being biotinylated horse-antimouse immunoglobulin G at an incubation concentration of 1:100 with an incubation time of 30 minutes.12,28,34

**Direct Injection of rHuTNF-α Into Normal Brain**

To examine the possible local effects on brain tissue after injection of rHuTNF-α, we conducted a study in normal, nontumor-bearing rats. In six rats, 7.3 μg rHuTNF-α dissolved in 10 μl of 50 mM Na-P buffer (pH 8.5) was given in a single injection over 1 minute into the right caudate nucleus. Two other rats received 10 μl inactivated human serum to exclude local reaction to human protein.

**Results**

**Receptor Binding Assay**

Figure 1 shows the receptor binding assay results for the binding of $^{125}$I-rHuTNF-α to U937 cells (histiocytic human cell line) corrected for nonspecific binding. From Scatchard analysis, the estimated number of surface rHuTNF-receptors per cell was about 2500, the dissociation constant being $1.09 \times 10^{-10}$ M. Figure 2 demonstrates the binding curve for the RG-2 cells. The nonspecific binding curve is virtually identical to the total activity count for $^{125}$I-rHuTNF-α, thus indicating a total lack of TNF receptors.

**Growth Inhibition Assay**

No growth-inhibiting effects of rHuTNF-α on RG-2 cells could be observed in vitro. The effective concentration to achieve a 50% inhibition (EC$_{50}$) of $^{3}$H-TdR incorporation was 0.005 μg/ml rHuTNF for the U937 cell line, but was found to be greater than 10$^2$ μg/ml for the RG-2 cells. Even at a concentration of 20 μg/ml rHuTNF, no significant reduction of $^{3}$H-TdR incorporation into proliferating tumor cells could be observed.

**Pharmacokinetics**

The serum concentration of rHuTNF-α equivalents in RG-2 glioma-bearing rats after intravenous injection is shown in Fig. 3. The median (± standard error) serum concentration after 15 minutes was 16.89 ± 2.29 ng/ml.

The tissue concentration of rHuTNF-α equivalents in the liver, kidneys, heart, and lung of RG-2 glioma-
above. There was no statistical difference in the extent of necrosis between the TNF-treated animal group and the control animals. In addition, a blind histological analysis by two different examiners showed no difference between the two groups.

**Immunohistochemical Examination**

Immunohistochemical studies with monoclonal antibody to rHuTNF-α showed total disappearance of the substance after 24 hours. At the stereotactic canal up to 2 hours after injection of 10 μl (7.3 μg) rHuTNF-α, we saw strong immunoreactivity but no internalization of the factor into the cells. A mild leukocytotactic effect with monocytic and lymphocytic infiltration was noted 1 day after injection, but apart from this no specific effects could be observed; in particular, no necrotizing effect on the tumor cells was seen.

**Direct Injection of rHuTNF-α Into Normal Brain**

The stereotactic injection track was examined in serial frontal sections, stained with hematoxylin and eosin and with KV. Marked increased cell density could be seen at the wall of the track in all animals that had received rHuTNF-α. This increased cell density was due to proliferation of microglial elements as well as lymphocytes. In addition, microvascular thrombosis could be seen (Fig. 9 right). No such changes were observed in the control rats, which had been injected with only human serum (Fig. 9 left).

**Discussion**

The goal of these experiments was to investigate the possible therapeutic effects of rHuTNF-α on glioma cells, regardless of the presence or absence of specific binding sites on the cell surface. A variety of human and animal tumor cell lines (HeLa, NG108, UC) contain TNF receptors, usually ranging from 500 to 10,000 per cell. It was recently shown that after horizontal transfection with an Epstein-Barr virus-derived circulating DNA vector, TNF-receptor expression could be amplified to a receptor density of approximately...
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150,000 per cell. Although little is known at present about the exact mechanisms of post-receptor events leading to the production of intracellular messengers and finally cell death, it can be assumed that the cytotoxic effects of rHuTNF are in general directly receptor-mediated. However, as several authors have reported indirect (immunomodulatory/inflammatory-mediated) effects on tumor growth in vivo and in vitro, we decided to examine these effects in a well-described rat glioma cell line and a standardized rat glioma model. We believe that the use of this rat model system is an appropriate alternative to the use of nude mice-human tumor xenografts; the responsiveness of rat tissue to rHuTNF-α has been well documented previously and the antitumor activity of the substance is known to be species-nonspecific.

Fig. 7. Photomicrograph showing an RG-2 glioma 7 days after stereotactic injection of 10,000 cells into the right caudate nucleus.

Fig. 8. Histological appearance of implanted tumor 14 days after inoculation showing necrotic tumor cells and large phagocytosing mononuclear inflammatory cells. H & E. × 120.

Fig. 9. Photomicrographs of sections showing injection tracks in normal brain. KV stain, × 71. Left: After injection of 10 μl heat-inactivated human serum, hemorrhage into the injection track is visible but there is only mild perifocal reaction. Right: After injection of 7.3 μg rHuTNF-α there is marked microglial and mononuclear inflammatory cell reaction as well as microvascular thrombosis.
Our experiments could not prove any therapeutic effect in the RG-2 glioma model, either after application in vitro or after stereotactic injection. Although extensive necroses could be observed in some tumors, there was no difference in tumor size between treated and nontreated animals. The large necroses observed in some of the tumors are due to other factors, which are currently unknown. However, one could have expected some therapeutic effects even in receptor-negative tumors from the marked chemotactic effects of rHuTNF-α. These chemotactic effects have recently been extensively investigated by various authors.\(^5,44,46\)

Chemokinetic effects of rHuTNF-α on human monocytes were shown in concentrations as low as 0.7 pg/ml.\(^21\) Other authors, however, have pointed to the complexity of the biological reactions caused by rHuTNF-α in vitro and in vivo and have shown a concentration-, time-, and place-dependent action.\(^31\) They have suggested that, in vitro, neutrophils and monocytes available for participation in an antitumor activity are only those present in the tumor at the outset. Because the RG-2 gliomas have a uniform morphology without cellular reactions on Day 7 (the date of treatment in our experimental setting) and because necrosis tends to occur at a later stage in tumor growth, it is not surprising that no beneficial effect was observed after stereotactic treatment with rHuTNF-α. Systemic application of rHuTNF-α resulted in increased concentration of the substance in the gliomas compared to that in brain adjacent to the tumor and the contralateral brain. This increased concentration can be explained by the disrupted blood-brain barrier of the experimental glioma, as there was no true internalization of the factor into the cells shown by immunohistochemistry.

Marked effects of rHuTNF-α were seen after stereotactic injection into normal rat brain; these consisted of a strong inflammatory response surrounding the injection track. This inflammatory response must be explained by a strong leukocytotoxic effect and not by a general inflammatory response caused by injection of human protein, because the control brains, injected with inactivated human serum, showed no marked cell reaction. In addition, capillary microthrombosis was noted in the experimental specimens, a biological rHuTNF-α effect well known from previous examinations.\(^38\)

Conclusions

From our experimental glioma data, we conclude that brain tumors without rHuTNF-α receptors neither respond to direct antitumor effects of the drug nor seem to show indirect immunomediated therapeutic benefit after stereotactic intratumoral delivery. Therefore, before clinical use of rHuTNF-α in malignant glioma patients, receptor binding studies should be performed on early tumor cell cultures. In receptor-positive patients who might be candidates for stereotactic treatment to avoid systemic side effects, one should carefully prevent rHuTNF-α leaking into normal brain. If exact positioning of the needle in the tumor cannot be achieved, severe local encephalitic reactions might be anticipated.

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