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Detection of tumor necrosis factor-α protein and messenger RNA in human glial brain tumors: comparison of immunohistochemistry with *in situ* hybridization using molecular probes

KARL ROESSLER, M.D., GERDA SUCHANEK, PH.D., HELENE BREITSCHOPF, PH.D., KLAUS KITZ, M.D., CHRISTIAN MATULA, M.D., HANS LASSMANN, M.D., AND WOLFGANG TH. KOOS, M.D.

Department of Neurosurgery, University Hospital of Vienna, Austria; and Research Unit for Experimental Neuropathology, Austrian Academy of Sciences, Vienna, Austria

Tumor necrosis factor-α (TNFα) protein and messenger (m)RNA distribution was studied in biopsy samples of glial brain tumors, using immunohistochemistry and *in situ* hybridization with molecular probes, to investigate the role of this cytokine in tumor proliferation and immunological host defense. Focal expression of TNFα was detected in four of four glioblastomas, one of two anaplastic astrocytomas, and four of five low-grade astrocytomas, regardless of their subtype or grade of malignancy, but in none of the normal peritumoral brain tissues used as controls. The TNFα protein and mRNA were present in reactive astrocytes and protoplasmic tumor cells, confined to areas of leukocyte or T-lymphocyte infiltration, and less pronounced in tumor cells at the edge of necrosis. Additionally, TNFα reactivity was found in infiltrating macrophages and perivascular microglia. Immunohistochemistry and *in situ* hybridization for TNFα showed comparable reaction patterns and numbers of TNFα-positive cells, even though the sensitivity of *in situ* hybridization was significantly higher. Quantitative evaluation of TNFα protein, TNFα mRNA, and leukocyte infiltration revealed a significant positive correlation between the TNFα-positive reactive astrocytes and the number of lymphocytes present in corresponding areas. Together, these data lead to the conclusion that TNFα in reactive astrocytes and monocytic cells within tumor areas of high leukocyte infiltration and in tumor cells at the border of necrosis may represent one defense pathway of the immune system against tumor proliferation.

KEY WORDS • tumor necrosis factor-α • glioma • cytokine • immunotherapy • immunohistochemistry • *in situ* hybridization

THE 17-kD molecular weight cytokine tumor necrosis factor-α (TNFα) seems to play a key role as an immunoregulatory molecule in various neurological diseases.[4,7,14,15,18,20,21,26,29,31,41,42] Its antiproliferative and cytotoxic activity has been demonstrated in both *in vivo* and *in vitro* studies,[8,19,35,37] but its role in immune response of glioma growth, whether antiproliferative or mitogenic, is still open to debate.[3,9,15,24,38,40] In isolated central nervous system (CNS) tissue cultures, astrocytes,[10–12,20,27,39,44] microglial cells,[10,39] and glioma cells[3] were shown to have the ability to secrete TNFα on stimulation by different cytokines. Although immunohistochemical studies of glioma tissue indicate an interaction of glial brain tumor cells with resident brain cells and inflammatory leukocytes,[13,34,] the role of cytokines, and in particular TNFα, in this process is not clear.[2,5,12,39] Thus, we have investigated the site of TNFα protein and TNFα messenger (m)RNA expression by using immunohistochemistry and *in situ* hybridization with molecular probes in various glial brain tumors.

Materials and Methods

Tissue Samples

Fresh biopsy tissue from 11 patients with glial brain tumors was obtained during neurosurgical procedures at the Department of Neurosurgery, University Hospital of Vienna, Austria (Table 1). Two samples of peritumoral brain tissue that had no pathological changes served as controls. Diagnosis was confirmed by neuropathological examination of corresponding paraffin-embedded tissue at the Neurological Institute, University Hospital of Vienna, Austria.

Tissue Processing

Biopsy specimens were snap-frozen in liquid nitrogen. Cryostat sections (5 μm thick) were cut, air-dried for 30 minutes at room temperature, fixed in acetone for 5 minutes, dried again, and stored at −75°C until use.

Antibodies, Cytokines, and Molecular Probes

Table 2 gives the results of tests conducted using the antibodies, cytokines, and molecular probes described below. Both murine monoclonal antibodies against TNFα (Ing. Brückner, Olympus,

TABLE 1

Results of biopsy in 11 patients with glial brain tumors

Case No.	Age (yrs), Sex	Pathological Diagnosis	Grade*	Mean Ki 67 Index (%)
1	41, M	subependymoma	I	<1
2	53, F	ependymoma	I	1.2
3	24, M	astrocytoma	I	1.5
4	2, M	astrocytoma	I	1.6
5	14, F	pilocytic astrocytoma	I	<1
6	5, M	astrocytoma	II	2
7	38, F	anaplastic astrocytoma	III	8.5
8	6, F	glioblastoma multiforme	IV	18.3
9	64, F	glioblastoma multiforme	IV	22.6
10	69, M	glioblastoma multiforme	IV	15.8
11	14, M	glioblastoma multiforme	IV	25.5

* Grade according to Kernohan Scale (Kernohan JW, Mabon RF, Svien HJ, *et al*: Symposium on new and simplified concepts of the gliomas. A simplified classification of the gliomas. *Proc Staff Meet Mayo Clin 24:*71–75, 1949).

TABLE 2

*Antibodies, cytokines, and molecular probes used to detect TNFα protein**

Name	CD	Dilution	Reactivity	Source
aCLA	CD45	1:100	CLA	Dakopatts
Leu4	CD3	1:100	T cells	Becton Dickinson
aT cell polycl	CD3	1:100	T cells	Dakopatts
aGFAP	—	1:100	GFAP/ astrocytes	Boehringer Mannheim
VIM12	CD11b	1:200	CR3/ macrophages	Knapp, *et al.*, 1984
MRP14 polycl	—	1:10000	L1 protein, macrophages	Odink, *et al.*, 1987
MAb Ki 67	—	1:20	proliferating cells	Dakopatts[16]
ahuTNFa monoclonal	—	1:200	αhuTNFα	Olympus
αhuTNFα monoclonal	—	1:100	αhuTNFα	Bender Vienna
αhuTNFα polycl	—	1:10000	hαhuTNFα	Genzyme
recombinant TNFα	—	ND	mono/polycl TNFα-antibodies	Genzyme
TNFα oligoprobe	—	ND	antisense oligonucleotides specific for Exon 3, 4A, 4B	British Bio-Technology
TNFα oligoprobe	—	ND	oligonucleotides specific for TNFα†	Sandoz, Dr. Bevec
TNFα cDNA probe	—	ND	1.59-kb cDNA probe specific for TNFα	ATCC

* TNF = tumor necrosis factor; CD = cluster designation; aCLA = antibody to common leukocyte antigen; polycl = polyclonal; aGFAP = antibody to glial fibrillary acidic protein; — = no cluster designation name defined; CR3 = complement receptor 3; MAb = monoclonal antibody; αhuTNFα = human recombinant TNFα; ND = not diluted; cDNA = complementary DNA; ATCC = American Type Culture Collection.

† As specified by the producer, Dr. Bevec.

Vienna, Austria, and Dr. Adolph, Bender, Vienna, Austria) and the rabbit polyclonal anti-TNFα (Genzyme Corp., Cambridge, MA) were titrated on the phytohemagglutinin-stimulated U937 cell line to determine the optimum concentrations. The following antibodies are routinely used in our laboratory and were tested on a wide variety of tissue to determine specificity and optimum concentration: common leukocyte antigen (CLA; Dakopatts, Copenhagen, Denmark); antilymphocyte (Leu4, monoclonal; Becton Dickinson, Mountain View, CA; and anti–T cell, polyclonal; Dakopatts); antimacrophage (VIM12; Dr. Knapp, Institute of Immunology, Vienna, Austria; and MRP 14 polyclonal; C. Sorg, Institute of Experimental Immunology, University of Muenster, Muenster, Germany); antiglial fibrillary acidic protein (GFAP, monoclonal and polyclonal; Boehringer Mannheim, Mannheim, Germany), and Ki-67 monoclonal antibody (Dakopatts). Irrelevant antibodies were used as controls for monoclonal and polyclonal antibodies. Additionally, recombinant TNFα (Genzyme) was used successfully for absorption of anti-TNFα antibody activity. For *in situ* hybridization, two TNFα oligoprobes (British Bio-Technology, Oxford, UK; Dr. Bevec, Sandoz, Vienna, Austria) and a TNFα complementary (c)DNA probe (American Type Culture Collection, Rockville, MD) were used.

Immunohistochemical Staining Procedures

Immunohistochemical staining of frozen tissue sections was performed as described earlier.[36] For the detection of primary antibodies, a direct peroxidase as well as an alkaline phosphatase method using enzyme-conjugated $F(ab')_2$ fragments (Immunotech, Marseille, France) as secondary antibodies were used. Visualization was performed with diaminobenzidine (DAB; Fluka Chemicals, Buchs, Switzerland) or with Fast Blue BB Salt (FBS; Sigma Chemical Co., St. Louis, MO), respectively. For double-staining procedures a mouse monoclonal first primary antibody was detected with a peroxidase-conjugated $F(ab')_2$ fragment and visualized with DAB (brown precipitate). Subsequently, a rabbit polyclonal second primary antibody was incubated and detected with the species-specific alkaline phosphatase–conjugated $F(ab')_2$ fragment and developed with FBS medium to avoid immunological cross reaction and nonspecific staining.

In Situ *Hybridization*

Detection of TNFα mRNA was performed on paraffin sections of corresponding areas using two different oligoprobes and a TNFα-specific 1.5-kb cDNA probe. *In situ* hybridization was performed with a nonradioactive system, following the protocol of Breitschopf, *et al.*[6] The temperature for hybridization and stringency was slightly modified because of the use of oligo- and cDNA probes. Briefly, 5-μm-thick sections of paraffin-embedded tissue were carefully dewaxed, treated with proteinase K (Sigma), and incubated with digoxigenin-labeled probes specific for TNFα. Oligoprobes were labeled by tailing and were used as well as cDNA probes labeled by random priming, both according to the manufacturer's instructions. After a stringent washing step, the reaction was followed by incubation with alkaline phosphatase–labeled antidigoxigenin antibody. The color reaction was obtained using FBS.

Enzyme-Linked Immunosorbent Assay System and Northern Blotting

These experiments were performed to define the specificity of anti-TNFα antibodies and probes used in the study. The TNFα production was measured in phosphomolybdic acid (PMA)-stimulated U937 myelomonocytic cells (D. Kraft and O. Förster, Institute of Experimental Pathology, Vienna, Austria) using cytospins and a sandwich enzyme-linked immunosorbent assay system. Briefly, microtiter plates were coated with murine monoclonal anti-TNFα (500 ng/ml, Bender) and incubated with twofold serial dilutions of cell culture supernatants or cell lysates. Thereafter, plates were sequentially exposed to rabbit anti-TNFα (1:1000), horseradish peroxidase–conjugated goat anti–rabbit antibody, with 3,3′,5,5′tetramethylbenzidine dihydrochloride as substrate. Human recombinant TNFα served as standard, with a detection limit of 10 pg/ml. Total U937 RNA was extracted by the standard thiocyanate extraction procedure, followed by electrophoretic separation of total RNA (15 μg/lane) on formaldehyde agarose gel, and subsequent blotting onto a nylon membrane. Ribosomal RNA bands 28S and 18S served as

TABLE 3

*Tumor necrosis factor-α protein and messenger RNA in glial brain tumors of 11 patients**

Case No.	Diagnosis & Grade	TNFαIR+ Stellate Cells†	TNFαIR+ Tumor Cells†	TNFα mRNA†	Inflammation‡	Necrosis§
1	subependymoma I	−	−	+	−	−
2	ependymoma I	+++	−	+++	++	−
3	astrocytoma I	+	−	−	+	−
4	astrocytoma I	−	−	+	+	−
5	astrocytoma I	+	−	++	++	−
6	astrocytoma II	+	−	++	+	−
7	astrocytoma III	+	−	+	+	−
8	glioblastoma IV	+	+	+++	+	+
9	glioblastoma IV	++	+	+++	++	+
10	glioblastoma IV	++	++	+++	++	+
11	glioblastoma IV	+	+++	+++	++	+

* TNF = tumor necrosis factor; mRNA = messenger RNA; IR = immunoreactivity; TNFαIR+ = TNFα-positive.

† − = TNFα negative; + = less than 10 positive cells/10 mm^2; ++ = between 10 and 20 positive cells/10 mm^2; +++ = more than 20 positive cells/10 mm^2.

‡ − = less than 10 cells/10 mm^2; + = between 10 and 100 positive cells/10 mm^2; ++ = more than 100 positive cells/10 mm^2.

standards. Hybridization was performed with ^{32}P-labeled oligonucleotide probes and cDNA, respectively. Hybridized radioactivity was visualized by exposure to Kodak XAR-5 film and intensifying screen at −70°C for various time periods. There was an increase of TNFα production in the first 6 hours of cultivation, which leveled off after 24 hours. In a typical experiment, 730 pg TNFα production per million cells was found after 24 hours. After 1 hour of incubation, TNFα mRNA was found to be upregulated in PMA-stimulated U937 cells compared to unstimulated controls.

Quantitative Evaluation and Statistical Analysis

The K67+, TNFα, CLA+, and CD3+ cells were counted on adjacent sections of investigated tumor specimens. Numbers given in the figures reflect mean counts of positive cells in an area of 10 mm^2. An Apple computer (Cricket Graph) was used for statistical analysis and graphic presentation; a p value of less than 0.05 was considered statistically significant.

Results

Glial brain tumor biopsy specimens from 11 patients (four glioblastomas, proliferation index 15.8%–25.5%; one anaplastic astrocytoma, proliferation index 2%–8.5%; and six low-grade astrocytomas, proliferation index 0.5%–2%) were investigated by single and double immunohistochemistry and by *in situ* hybridization for TNFα protein and mRNA distribution to correlate leukocyte infiltration. Leukocyte infiltration within the tumor specimen detected by antibodies against CLA was high in three of four glioblastomas, but also in two low-grade gliomas (> 10 positive cells/mm^2) (Table 3). Other low-grade gliomas showed moderate numbers of infiltrating leukocytes (between one and 10 positive cells/mm^2) but one low-grade astrocytoma had almost no leukocyte infiltration (< one positive cell/mm^2) (Table 3). The percentage of lymphocytes as determined by anti-CD3 antibodies in these infiltrates was high in all investigated tumor biopsies (between 46% and 78%, mean 62%), except the one low-grade astrocytoma which had few intratumoral leukocytes that consisted of 14% lymphocytes (Fig. 1).

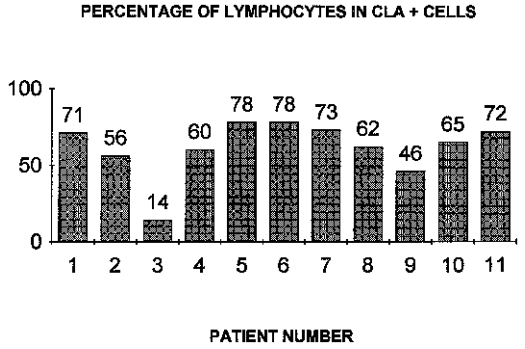

FIG. 1. Bar graph showing percentages of lymphocytes in CLA+ cells in 11 patients with glial brain tumors.

As detected by three different anti-TNFα antibodies and three different TNFα DNA molecular probes, TNFα-protein and mRNA expression showed a comparable reaction pattern and numbers of TNFα-positive cells in corresponding areas, although the sensitivity of *in situ* hybridization was higher. As protein, TNFα was detected in nine of 11 and TNFα mRNA in 10 of 11 specimens (Table 3), with sparse distribution within the biopsies. Quantitative evaluation showed less than one TNFα protein–positive cell per square millimeter in six cases, between one and two positive cells per square millimeter in two, and more than two positive cells per square mil-

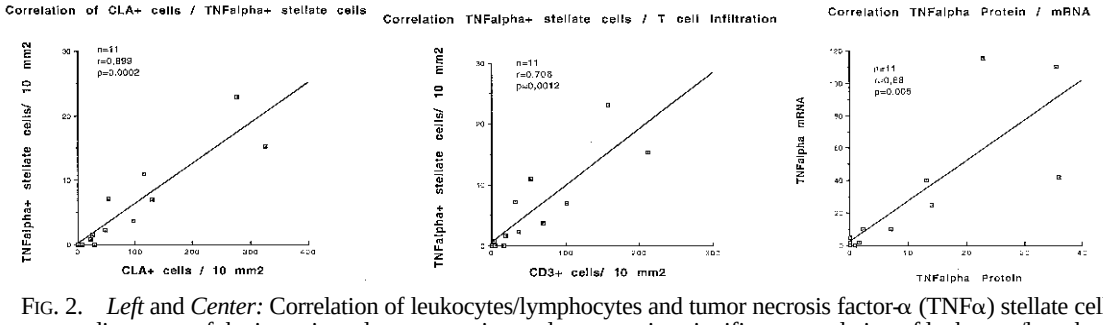

FIG. 2. *Left* and *Center:* Correlation of leukocytes/lymphocytes and tumor necrosis factor-α (TNFα) stellate cells in corresponding areas of the investigated tumor specimen, demonstrating significant correlation of leukocyte/lymphocytes and TNFα expression in immunoactivated areas. *Right:* Significant correlation of TNFα messenger RNA and TNFα protein immunoreactivity in corresponding areas, demonstrating the reliability of the *in situ* hybridization method.

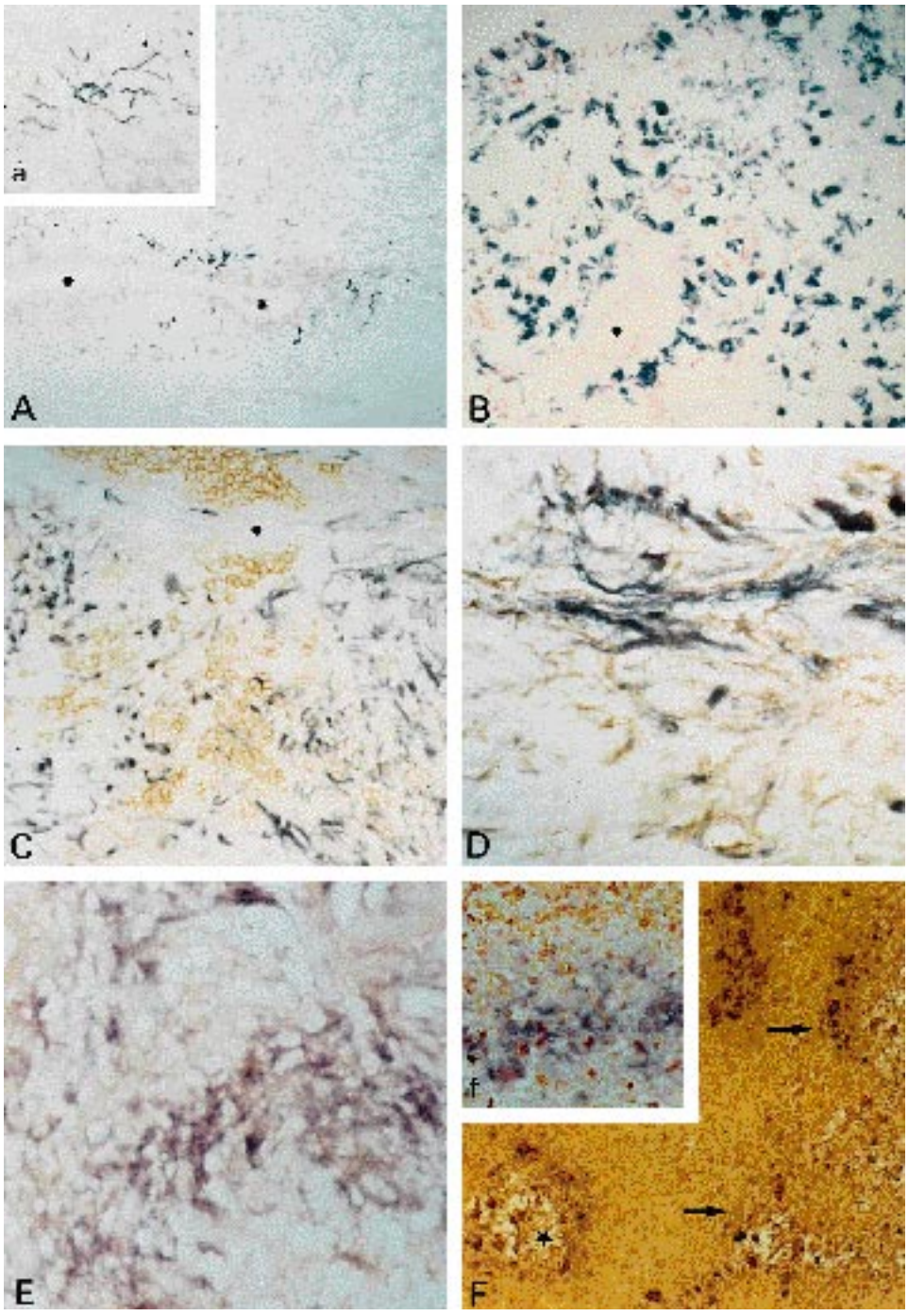

FIG. 3. Photomicrographs of glial cells showing immunohistochemical (A to D) and *in situ* hybridization (E and F) studies. A: Expression of tumor necrosis factor-α (TNFα) in a small number of stellate cells in areas of sparse leukocyte infiltration within a low-grade glioma, intraparenchymally *(inset)*, and in perivascular position. Vessel lumen is indicated by *asterisks*; *arrow* shows lymphocyte in close contact with TNFα-positive astrocytic processes. Double immunostaining with anti-TNFα (blue)/common leukocyte antigen (yellow): combination of alkaline phosphatase–peroxidase technique. Original magnification × 400 (A); × 640 *(inset)*. B: Large, polymorphic, anaplastic tumor cells expressing TNFα protein (blue) in a glioblastoma. The endothelial cells around the center of the vessel lumen *(asterisk)* are TNFα-negative. Alkaline phosphatase staining/nuclear fast red, original magnification × 640. C: High numbers of infiltrating T cells (anti-CD3) surrounded by a network of TNFα-positive stellate cells. Double immunostaining with anti-TNFα (blue)/antilymphocyte (CD3, yellow) in areas with high lymphocyte infiltration: combination of alkaline phosphatase–peroxidase staining, original magnification × 640. D: Double immunohistochemistry with anti-TNFα protein (blue) and anti-glial fibrillary acid protein (GFAP) (yellow) identifying most of the TNFα-positive cells as astrocytes. Alkaline phosphatase–peroxidase staining, original magnification × 800. E: Low-grade glioma, paraffin section, showing *in situ* hybridization for TNFα messenger (m) RNA. A network of TNFα mRNA (blue) containing stellate cells is revealed within a low-grade glioma, similar to findings on immunohistochemistry (C and D). Alkaline phosphatase–digoxigenin-labeled specific DNA probe for TNFα, original magnification × 400.

Continued→

limeter in one, whereas TNFα mRNA was detected in five cases, in more than 2 cells per square millimeter in each case. Quantitative assessment of TNFα protein and TNFα mRNA–positive cells showed a significant positive correlation ($r = 0.68$, $p = 0.005$) in corresponding areas (Fig. 2 *right*).

For the most part, TNFα reactivity was found in two different cell populations, mainly in stellate cells within the tumor and peritumoral tissue, with a few cells appearing in perivascular locations (Fig. 3A, C, and E). In addition, TNFα protein and mRNA were present in large, polymorphic, anaplastic cells located at the border of necrotic areas in the four cases of glioblastomas investigated (Fig. 3B and F). In these four glioblastomas, TNFα mRNA/TNFα protein–positive tumor cells were exclusively detected around necrotic tumor areas, whereas TNFα mRNA/TNFα protein–positive stellate cells with no connection to necrosis were found in low- and high-grade tumors.

Double immunohistochemistry, first with anti-TNFα antibodies and molecular probes, then with GFAP and macrophage marker (anti-CD11b and MRP14), identified most of the TNFα-positive stellate cells as TNFα/GFAP double-positive reactive fibrous astrocytes (Fig. 3D), and a few of them as TNFα/MRP14-positive macrophages or microglia. Nearly all TNFα-positive large protoplasmic cells in the border of necrotic areas were demonstrated as TNFα/GFAP double-positive tumor cells; a few of them were identified as TNFα/VIM12-positive macrophages (Fig. 3F *inset*). Double-staining experiments with anti-TNFα as first step and anti-CD3/anti-CLA antibodies as second step demonstrated the limits of sparsely distributed TNFα-positive stellate cells to areas of high leukocyte–lymphocyte infiltration (Fig. 3C). Statistical analysis of detected numbers of leukocyte–lymphocyte infiltrates compared to numbers of TNFα-positive stellate cells within these corresponding tumor areas also revealed significant correlations ($r = 0.706$, $p = 0.0012$ for CD3+ cells; $r = 0.899$, $p = 0.0002$ for CLA+ cells) (Fig. 2 *left* and *center*). No significant correlation was found between the numbers of positive TNFα mRNA/TNFα protein–positive cells and the proliferation index within the investigated low- and high-grade tumors.

Discussion

This study reveals a role for TNFα in the local immune response in human glial brain tumors *in vivo*. We have demonstrated the occurrence of a TNFα mRNA and TNFα protein–positive cell population directly correlated with the number of infiltrating lymphocytes in glial cell brain tumors, and we have characterized TNFα-producing and -secreting cell types and their location in biopsy tissue of 11 patients, comparing immunohistochemical and *in situ* hybridization methods.

Production of TNFα by Reactive Astrocytes, Microglia, and Macrophages

The presence of inflammatory leukocyte infiltrates in glial brain tumors is well known from previous studies and suggests an ongoing immune response within the tumor tissue.[13,34] The cellular subsets of infiltrating lymphocytes are well characterized,[23] but the interaction of these immune cells and tumor cells *in vivo* is not clearly understood. Studies of regulatory mechanisms found a predominant role for the cytokine TNFα.[24,40]

In our study, TNFα was shown to be produced and secreted by reactive astrocytes located mainly in areas with lymphocytic inflammation. This finding confirms *in vivo* studies of isolated CNS tissue cultures, in which astrocytes were demonstrated to have the capacity to secrete TNFα in response to inflammatory stimuli.[10,11,28,35,39] Additionally, we were able to identify single TNFα-reactive glia in tumor and peritumoral tissue as well as perivascular locations in low- and high-grade gliomas.

Expression of TNFα was not limited to reactive astrocytes; in additional double-staining experiments we characterized two other cell types as TNFα secreting within the investigated gliomas. Few perivascular microglial cells and macrophages were identified as TNFα positive. Macrophages were believed to be the principal source of TNFα;[32] however, recently it was discovered that TNFα is secreted by a variety of cell populations in the course of microbial infections, neoplastic disease, and autoimmune disorders.[32] In addition, microglial cells are known as the major producers of TNFα in the brain.[17,39]

Production of TNFα by Anaplastic Glial Tumor Cells

In addition to reactive astrocytes, large anaplastic tumor cells around necrotic areas of the glioblastomas investigated were shown to contain TNFα mRNA and TNFα protein. In earlier *in vitro* studies, the ability of different glioma cell lines to produce TNFα and to express TNFα receptor was shown.[3,37] Although TNFα is known for its cytotoxic and cytostatic effect against various tumor cell lines,[8,25,43] its general effect on glial tumor cells *in vivo* and even *in vitro* is not clear. Although TNFα was mitogenic for human astrocytes[2] and the U373 astrocytoma cell line,[24] antiproliferative and cytotoxic effects of this factor in two glioma cell lines were described.[37]

In our study the TNFα-positive tumor cells were found only in the four cases of glioblastoma investigated, not in anaplastic or low-grade astrocytomas. Also, we detected TNFα in polymorphic tumor cells located only at the border of necrotic tissue areas, which strongly indicates an *in vivo* involvement in the formation of tumor necrosis, a well-known histological hallmark of malignant gliomas. Moreover, the ability of TNFα to induce tumor tissue necrosis is well known from previous *in vitro* studies.[8]

F: Paraffin section showing *in situ* hybridization for TNFα mRNA (blue), secondarily immunostained with GFAP (brown). Anaplastic tumor cells at the border of necrotic areas *(star)* showing TNFα mRNA detection in a glioblastoma *(large arrows)*. Tumor cells were double stained with TNFα mRNA/GFAP alkaline phosphatase–peroxidase double-staining technique. Original magnification × 640. *Inset:* A secondarily anti-CD3 (brown) immunostained paraffin section counterstained with a nuclear fast red: results of *in situ* hybridization for TNFα mRNA (blue, visualization of alkaline phosphatase–digoxigenin-labeled specific DNA probe) in a glioblastoma with TNFα mRNA-positive tumor cells at the border of a necrotic tumor focus showing high lymphocyte infiltration. Alkaline phosphatase–peroxidase technique/nuclear fast red, original magnification × 400.

Correlation of TNFα With Tumor-Infiltrating Lymphocytes

By analyzing the staining pattern of TNFα-positive cells and inflammatory infiltrates in sparsely distributed areas within the tumor tissue, we were able to demonstrate a highly significant positive correlation between TNFα-reactive astrocytes and the number of lymphocytes in the corresponding areas. Therefore, our study finds that an active immune response takes place between lymphocytes and glial cells within these tumor areas. Paracrine cytokine loops involving resident immunocompetent cells, leukocytes, and tumor cells are conceivable. Cytokines may permit leukocytes to invade the CNS tissue via upregulation of endothelial adhesion molecules like ICAM-1 and LFA-3,[36] or they may interact in a complex network to control immune defense cells when they respond to antigen.[1] Similar TNFα-mediated immunoreactions in the CNS were shown for multiple sclerosis,[20] and viral, bacterial, and protozoan infection.[20,26,30,42]

Conclusions

Our study suggests the involvement of the cytokine TNFα in the immune response against low- and high-grade human gliomas. In addition, the expression of TNFα in tumor cells at the edge of necrosis indicates that this cytokine is involved in the destruction and elimination of tumor cells.

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Address reprint requests to: Karl Roessler, M.D., Department of Neurosurgery, University Hospital of Vienna, Waehringer Guertel 18–20, A-1090 Vienna, Austria.