Distribution of endogenous tumour necrosis factor α in gliomas

M Maruno, J S Kovach, P J Kelly, T Yanagihara

Abstract

Aims—To determine the distribution and cellular origin of endogenous tumour necrosis factor α (TNF α) in the cellular components of human gliomas.

Methods—Frozen sections of 26 gliomas (four astrocytomas (As); two oligo-astrocytomas (OA); one anaplastic astrocytoma (AA); one anaplastic oligo-astrocytoma (AOA); 18 glioblastomas (GB)) were examined immunohistochemically using antihuman TNF α and anti-Leu-M5 (CD11c) antibodies. Additional studies with double immunohistochemical procedures were performed with anti-glial fibrillary acidic protein and anti-neurofilament antibodies.

Results—Eighty per cent of the AA, AOA, and GB (16 of 20) had a positive reaction for TNF α , but only 17% of As and OA (one of six) were positive. Positive cells were seen in both the tumour tissue and adjacent brain tissues. TNF α protein was detected not only in the tumour cells but also in the endothelium of tumour vessels as well as reactive astrocytes and neurons.

Conclusions—Endogenous TNF α is present in cells of various origins in glial tumours including tumour vessels; however, the role of TNF α may be different in different types of cells or altered microenvironment.

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Keywords: endogenous tumour necrosis factor α ; gliomas; glioma cells; endothelial cells

Cytokines are essential mediators of cell to cell interactions in various pathophysiological processes. Tumour necrosis factor α (TNF α) is one of the major macrophage derived cytokines that was originally found to mediate cytostatic and cytotoxic effects against malignant cells in vitro, and to cause haemorrhagic necrosis in certain types of tumours in vivo.¹ TNF α possesses multiple biological and pleiotropic functions such as cytotoxicity, and promotion of cell differentiation and growth, as well as playing many immunomodulatory roles within the cytokine network.²⁻⁴

It is well known that TNF α exerts its biological activity by binding to specific cell surface receptors.⁴ Two distinct types of TNF receptors weighing 55 kDa (type I) and 75 kDa (type II) have been identified.⁵ Although, the distribution of TNF receptors in glioma tissues is obscure, there are few reports in the literature suggesting the distribution of TNF α

and endogenous TNF α possession by various glioma cell types.⁶ Hence, we examined immunohistochemically the distribution of endogenous TNF α in human glioma tissues

Materials and methods

Twenty six glioma samples were investigated (four astrocytomas (As); two oligo-astrocytomas (OA); one anaplastic astrocytoma (AA); one anaplastic oligo-astrocytoma (AOA); 18 glioblastomas (GB)). Histological diagnosis was performed according to the WHO classification.⁷ Immediately after surgical removal, the samples were frozen in isopropyl alcohol cooled with dry ice and stored at -80°C . Each sample was sectioned to 8 μm thickness in a cryostat microtome at -20°C , mounted on gelatin coated glass, dried at room temperature, and kept at -80°C until further processing.

For immunohistochemical procedures, the sections were fixed in 4% paraformaldehyde for five minutes, washed in 0.01 M phosphate buffered saline (PBS) and then immersed in 60% and 100% methanol. After washing in PBS, non-specific protein binding was blocked by incubation in 1.5% normal goat serum in

Table 1 Immunohistochemical study of human gliomas for TNF α and monocyte/macrophage staining

Specimen	Histology of glioma	TNF α		Monocyte/macrophage
		Tumour	Brain	
1	AS	-	NA	++
2	As	-	NA	++
3	As	+	NA	++
4	As	-	-	+++
5	OA	-	NA	+
6	OA	-	NA	+++
7	AA	-	NA	++
8	AOA	+	++	+++
9	GB	+	+	+++
10	GB	+	NA	+++
11	GB	+	NA	++
12	GB	-	+	+++
13	GB	++	++	+++
14	GB	+	NA	+++
15	GB	+	NA	+++
16	GB	+	NA	+++
17	GB	+++	NA	++
18	GB	+	NA	++
19	GB	-	NA	++
20	GB	++	NA	++
21	GB	++	NA	++
22	GB	-	+	++
23	GB	+	NA	++
24	GB	+	NA	++
25	GB	+	NA	++
26	GB	+	++	+++

Immunoreaction was arbitrarily categorised as: very frequent staining (+++), $\geq 20\%$ of the background cells; moderately frequent (++), $< 20\%$ of the background cells; only a few (+), $\leq 5\%$ of the background cells; and none (-), 0% cells.
As, astrocytoma; AA, anaplastic astrocytoma; GB, glioblastoma; OA, oligo-astrocytoma; AOA, anaplastic oligo-astrocytoma; NA, surrounding brain tissue not included in the specimen.

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Figure 1 (A) Immunostaining for TNF α in glioblastoma. Cytoplasm of most of the glioblastoma cells was positive for TNF α . Several cells show a strong positive reaction (original magnification $\times 400$). (B) Immunostaining for TNF α in surrounding brain tissue. Positive reaction for TNF α was present in the cytoplasm of numerous cells in the brain tissue adjacent to a glioblastoma (original magnification $\times 200$). (C) Positive reaction was completely abolished by using the antibody for TNF α after absorption with recombinant TNF α (original magnification $\times 200$). (D) Immunostaining for CD11c in glioblastoma. The monocytes/macrophages were seen moderately frequently (original magnification $\times 200$).

1% bovine serum albumin containing 0.2% Triton X100 for 20 minutes. The sections were then incubated overnight at 4°C with the primary antibody, rabbit polyclonal antihuman TNF α (1/1000) (a generous gift from Genentech, California, USA). Normal rabbit serum was used in lieu of anti-TNF α at the same dilution as negative control. Further incubation for 30 minutes with biotinylated antirabbit IgG and 30 minutes with avidin-biotin complex conjugated with alkaline phosphatase (Vectastain; Vector, California, USA) was performed followed by a final incubation for 30 minutes with Vector Red (Vector). The cell nuclei were visualised by counterstaining with haematoxylin. For identifying macrophages, mouse monoclonal anti-Leu-M5 (CD11c, 3.3 μ g/ml) (Becton Dickinson, California, USA), specific for human monocyte/macrophage antigen, was used. This was followed by incubation in antimouse IgG, avidin-biotin peroxidase complex (ABC-PO) (Vectastain; Vector), and 3,3'-diaminobenzidine tetrahydrochloride (DAB). As negative control, normal mouse IgG was used in lieu of anti-Leu-M5 (CD11c) at the same protein concentration.

To investigate the cellular origin of TNF α , we performed double immunohistochemical

procedures to visualise TNF α and a cell marker in the same cell. After the chromogenic reaction with Vector Red for TNF α , the sections were washed in 0.1 M glycine HCl buffer (pH 2.2) for 60 minutes, in Tris HCl buffer for 30 minutes, and then incubated in 0.15% hydrogen peroxide in Tris HCl buffer for 10 minutes. After washing, the sections were incubated in 1.5% normal horse serum for 20 minutes and then separately incubated overnight at 4°C with mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Boehringer Mannheim, Indiana, USA), or mouse monoclonal anti-neurofilament (NF) antibody against 68 kDa monomer (Boehringer Mannheim). After washing, the sections were incubated in biotinylated horse antimouse IgG for 30 minutes, and then with ABC-PO for 30 minutes, and finally incubated with DAB and hydrogen peroxide, followed by counterstaining with haematoxylin.

Results

ENDOGENOUS TNF α

Eighty per cent of the AA, AOA, and GB (16 of 20) were positive for TNF α , but only 17% of As and OA (one of six) were positive (table 1). Cells with positive reaction for TNF α were present not only in the tumour tissues (fig 1A)

Figure 2 (A) Double immunohistochemical study for TNF α and GFAP. Three cells with positive reaction for both (red for TNF α and brown for GFAP) are shown in the centre. These cells were, thus, identified to be astrocytic in origin (original magnification $\times 400$). (B) Double immunohistochemical study for TNF α and NF. A cell in the centre with positive reaction for both (red for TNF α and brown for NF) was identified to be a neuron (original magnification $\times 400$).

but also in the adjacent brain tissues (fig 1B). The reaction was recognised predominantly in the cytoplasm and was evident in various types of cells including multinucleated cells, large pleomorphic cells, and small cells. Cells with a positive reaction were most numerous at the boundary of the tumour tissues. Furthermore, some endothelial cells of the vessels within tumours or adjacent brain were also weakly positive for TNF α . No reaction was found in control sections. In addition, the use of antibody for TNF α before incubation with recombinant TNF α (Boehringer Mannheim) resulted in disappearance of the positive reaction (fig 1C).

REACTION FOR MONOCYTES/MACROPHAGES

A variable number of cells had a positive reaction with anti-Leu-M5 (CD11c) in all the study samples (fig 1D, table 1). Sometimes, monocytes/macrophages were found to be par-

ticularly numerous within the tumour parenchyma and comprised over 20% of the cellular elements (table 1). However, no correlation was seen between the expression of endogenous TNF α and the level of monocyte/macrophage infiltration.

DOUBLE IMMUNOHISTOCHEMICAL REACTIONS

Six GB were examined with double immunohistochemical reactions for TNF α and GFAP. While some cells in tumour tissues had positive reactions for both (fig 2A), the majority showed a positive reaction for TNF α alone. Four GB were examined with double immunohistochemical reactions for TNF α and NF. Whereas, some cells with large cytoplasm, especially in the adjacent brain tissues, showed positive reactions for TNF α and NF simultaneously (fig 2B), most of them showed a positive reaction for TNF α only.

Discussion

It is well known that TNF α acts as a potent immunomodulatory molecule affecting the function of many cells involved in the immune response, such as T cells, B cells, neutrophils, monocytes, and macrophages.⁸⁻⁹ In the central nervous system (CNS), astrocytes can produce a variety of immunoregulatory molecules upon stimulation. These include interleukin (IL)-1,¹⁰ IL-3,¹¹ prostaglandin E,¹⁰ interferon α and β ,¹² IL-6,¹³ and TNF α .¹⁴⁻¹⁶ Another CNS cell, the microglia, can also be stimulated to secrete IL-1,¹⁷ TNF α ,^{15,16} and IL-6.¹³ However, the tumour cells and endothelial cells in the territory of tumour were shown to be the major targets of exogenous TNF α in human gliomas.¹⁸

The present study demonstrated that cells containing TNF α protein were more abundant in malignant gliomas (AA, AOA, GB) than in benign ones (As, OA). Cells containing TNF α protein have been detected in the brain tissues of patients with multiple sclerosis¹⁹ and subacute sclerosing panencephalitis,²⁰ but not in the normal brain.¹⁹ In Guillain-Barré syndrome, the serum concentration of TNF α is raised, with a good correlation between TNF α and severity of the disease.²¹ Although TNF α was shown to be produced and secreted by reactive astrocytes located mainly in areas with lymphocytic infiltration,⁶ we could not find any correlation between the presence of TNF α protein positive cells and the number of infiltrating monocytes/macrophages in gliomas.

Macrophages were believed to be the principal source of TNF α , however, recently it was discovered that TNF α is secreted by a variety of cells during the course of microbial infections, neoplastic diseases, and autoimmune disorders.⁶ In addition, microglial cells are known to be the major producers of TNF α in the brain.^{16,22}

TNF α is also known to induce astrocyte proliferation in cultured cells,^{23,24} suggesting that it plays the role of growth factor producing prominent gliosis such as that found in a variety of CNS disease. The presence of cells con-

taining TNF α protein predominantly in the malignant gliomas substantiates the possibility that TNF α may be a growth factor for human gliomas.

The present study further demonstrated that vascular endothelium within gliomas and adjacent brain tissues were also positive for TNF α , although weakly. Vascular endothelium has been shown to be a direct target for the action of TNF α ,¹⁸⁻²⁵ modulating many endothelial cell functions such as the coagulant activity and immunological properties.²⁶⁻²⁸ In addition to these effects of TNF α , several other activities have been observed, such as alteration of the properties of endothelial cells, upregulation of major histocompatibility complex class I antigens, intercellular adhesion molecule-1, and other surface molecules that promote monocyte and neutrophil adherence to vascular endothelium. TNF α has also been shown to increase tissue factor procoagulant activity, enhance plasminogen activator inhibitor-1, and suppress expression of thrombomodulin, resulting in a net change of haemostatic properties of endothelium from an anticoagulant to a procoagulant state.²⁸ As TNF α may have a selective effect on the tumour vessels in gliomas,¹⁸⁻²⁵ this cytokine could possibly be an adjuvant to the treatment of malignant gliomas.

While our findings from double immunohistochemical procedures revealed that endogenous TNF α recognised in tumour tissues might have originated from the glial cells or neurons, the precise origin of TNF α in these cells could not be ascertained. A possibility exists that macrophages may produce TNF α , and the astrocytes and neurons may be the target cells for its binding.¹⁹ Based on the results of our double immunohistochemical procedures, there is a strong possibility that malignant glioma cells can produce TNF α . Although TNF α production on a per cell basis is lower compared with that by microglia or macrophages, a small but localised TNF α source has been recognised in astrocytes.¹⁶ It is anticipated that our findings of immunohistochemistry regarding the cellular origin of endogenous TNF α could be clarified by other techniques such as in situ hybridisation.

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