Expression of the neurotrophin receptors Trk A and Trk B in adult human astrocytoma and glioblastoma

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Neurotrophins and their receptors of the Trk family play a critical role in proliferation, differentiation and survival of the developing neurons. There are reports on their expression in neoplasms too, namely, the primitive neuroectodermal tumours of childhood, and in adult astrocytic gliomas. The involvement of Trk receptors in tumour pathogenesis, if any, is not known. With this end in view, the present study has examined 10 tumour biopsy samples (identified as astrocytoma, pilocytic astrocytoma and glioblastoma) and peritumoral brain tissue of adult patients, for the presence of Trk A and Trk B receptors, by immunohistochemistry. The nature of the tumour samples was also confirmed by their immunoreactivity (IR) to glial fibrillary acidic protein. In the peritumoral brain tissue, only neurons showed IR for Trk A and Trk B. On the contrary, in the tumour sections, the IR to both receptors was localized in the vast majority of glia and capillary endothelium. There was an obvious pattern of IR in these gliomas: high levels of IR were present in the low-grade (type I and II) astrocytoma; whereas in the advanced malignant forms (WHO grade IV giant cell glioblastoma and glioblastoma multiforme) the IR was very weak. These findings suggest that Trk A and Trk B are involved in tumour pathogenesis, especially in the early stage, and may respond to signals that elicit glial proliferation, and thus contribute to progression towards malignancy.

[Wadhwa S, Nag T C, Jindal A, Kushwaha R, Mahapatra A K and Sarkar C 2003 Expression of the neurotrophin receptors Trk A and Trk B in adult human astrocytoma and glioblastoma; *J. Biosci*. **28** 181–188]

1. Introduction

The development as well as maintenance of the nervous system is critically dependent upon members of a family of neurotrophins. Various neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6] are essential for the survival and maintenance of basal forebrain cholinergic neurons (NGF), dopaminergic striatal neurons and central GABAergic neurons (BDNF; Chao 1992; Barbacid 1994; Davies 1994; De la Rosa *et al* 1994; Bothwell 1995; Henderson 1996; Lewin and Barde 1996). Besides their role in neuronal development (Tucker *et al* 2001), several lines of evidence suggest a possible therapeutic potential for neurotrophins in pathology of the nervous system. For instance, axotomy- and ischemia-induced retinal changes in experimental animals can be delayed and transiently protected by intravitreal injections of NGF (Siliprandi *et al* 1993) and BDNF (Mansour-Roubaey *et al* 1994; Unoki and Lavail 1994). Neurotrophins mediate their various functions by binding to a low-affinity receptor, $r^{75\ NTR}$ (common to all neurotrophins) as well as to specific high-affinity receptors of tyrosine kinase family (Trk). NGF binds to Trk A, BDNF

Keywords. Astrocytoma; glioblastoma multiforme; immunohistochemistry; neurotrophin receptors; Trk A; Trk B

Abbreviations used: BDNF, Brain-derived neurotrophic factor; DAB, diaminobenzidine; DPX, dibutyl phthalate polystyrene xylene; GFAP, glial fibrillary acidic protein; H & E, hematoxylin and eosin; NGF, nerve growth factor; NT-3, neurotrophin-3.

and NT-4/5 bind to Trk B and NT-3 binds to Trk C (Klein *et al* 1991; Lamballe *et al* 1991; Chao 1992; Barbacid 1994). The binding of neurotrophins to their cognate receptors activates signal transduction by inducing autophosphorylation of the appropriate Trk receptors.

Studies have reported the expression of neurotrophin receptors in neuroblastoma, a type of pediatric tumour of the central nervous system (Donovan *et al* 1994; Nakagawara *et al* 1994). Washiyama *et al* (1996) demonstrated several neurotrophins as well as their receptors in medulloblastoma and other primitive neuroectodermal tumours of children. Trk receptors are expressed at high levels in these tumours, and the clinical significance thereof is suggested to be predictive of a favourable outcome (Segal *et al* 1994; Brodeur *et al* 1997; Dominici *et al* 1997). The available evidence tends to suggest that the Trk receptors greatly influence the clinical behaviour of the neoplasms.

Earlier, Wang *et al* (1998) had examined Trk A, Trk B and Trk C expression in 34 astrocytic tumours of different grades. We have conducted a similar study on examining the expression of Trk A and Trk B in adult human astrocytoma and noted that there is a clear difference in their pattern of expressions in early, benign to advanced malignant stages, not highlighted by them. Hence, this paper reports on the pattern of immunoreactivity to Trk A and Trk B receptors in adult astrocytoma and glioblastoma in surgical specimens.

2. Material and methods

2.1 *Sample collection*

Tumour tissues were obtained at surgery from 10 patients admitted to the Neurosurgery Department, All India Institute of Medical Sciences, New Delhi, for clinical treatment. Samples were taken from the main bulk of the tumours and the peritumoral region, which had to be excised during removal of the tumours. As diagnosed and confirmed by the surgeons, the peritumoral brain regions were without oedema or gross neuropathological changes. Details of the tumour types examined are summarized in table 1. Diagnosis as well as grading of the tumours was confirmed following the WHO classification (Kleihues *et al* 1993) by histological staining of sections with hematoxylin and eosin (H & E), and also by immunohistochemical staining for glial fibrillary acidic protein (GFAP).

2.2 *Tissue fixation*

All tissue materials were fixed in 4% paraformaldehyde for 24 h at 4°C. After rinsing, tissues were cryoprotected in 15% to 30% sucrose overnight and embedded in OCT compound (Miles, Elkhart, IN, USA). Frozen sections of 18–20 μm thickness were cut, serially collected in vials containing phosphate buffer (pH 7·4) and stored at 4°C until use. Adjacent sections (12 μm thickness) were mounted onto gelatin-coated slides and stained with H & E.

2.3 *Antibodies*

The Trk-receptor antibodies employed in this study were obtained from Santa Cruz Biotechnology, Inc. (California, USA). These were affinity-purified rabbit polyclonal antisera raised against (i) a peptide corresponding to 763–777 amino acid residues at the C-terminal domain of human Trk A (SC-118), and (ii) a peptide corresponding to 794–808 residues at the C-terminus of mouse Trk B (SC-12). Control peptides (SC-118P and 12P) were also procured from the same source. Both antibodies recognize the full-length isoforms of Trk receptors and the catalytic intracellular tyrosine kinase domain. According to the manufacturer, Trk A and Trk B receptor antibodies do not cross-react with each other, as determined by Western blot analysis.

2.4 *Immunohistochemistry*

Sections were initially treated with 0·3% hydrogen peroxide in 80% methanol to quench endogenous peroxidase activity. After repeated washing, these were incubated in 10% goat normal serum to block non-specific reactions. This was followed by incubation with the primary antibodies (dilution 1 : 2000) to Trk A and Trk B receptors for 48 h at 4°C. The secondary antibody used was biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, California, USA; 1 : 200 dilution, 6 h at 4°C). After washing, sections were put in avidin-biotin-pero xidase complex (Vector Laboratories; dilution 1 : 200)

Table 1. Details of the tumour samples examined.

Case No.	Age (year)	Sex	Tumour type and grade
1	15	M	Pilocytic astrocytoma II (cerebellar)
2	28	M	Protoplasmic astrocytoma II
3	32	M	Protoplasmic astrocytoma I
4	28	M	Pilocytic astrocytoma I (third ventricle)
5	35	M	Astrocytoma II
6	31	F	Giant cell glioblastoma IV
7	50	F	Glioblastoma multiforme IV
8	48	M	Glioblastoma multiforme IV
9	40	M	Glioblastoma multiforme IV
10	50	M	Giant cell glioblastoma IV

M, male; F, female.

for 3 h at room temperature. For visualization, sections were treated in 0·03% diaminobenzidine tetrahydrochloride (DAB) in 0·1 M acetate-imidazole buffer (pH 7·4) containing 0·5% nickel sulphate (as intensifier). Finally, sections were rinsed in water, mounted onto gelatin-coated slides, dehydrated in ethanol, cleared and covered with DPX mountant.

For immmunolabelling with GFAP, sections were incubated in a monoclonal mouse primary antiserum (Sigma Chemicals Co., St. Louis, MO, USA, catalog number G 3893, dilution 1 : 200) for 48 h, followed by incubation in biotinylated anti-mouse IgG (Vector Laboratories; 1 : 200 dilution, 6 h). The remaining procedure was identical, as outlined above.

As control, sections were incubated either without primary antisera or in the respective primary antisera preabsorbed with the control peptides (1 : 500) and then processed from the secondary antibody step onward, as described above.

Intensity levels of Trk immunoreactivity noted in individual tumour types were determined semiquantitatively with a four-point scale, where '1 → 4' denoted very weak to strong labelling, and are shown in table 2.

3. Results

In the apparently 'normal tissues' of peritumoral region, the immunoreactivity to Trk A and Trk B receptors was present in neurons only (figure 1b). Blood capillaries were nonreactive (figure 1c).

The pattern of Trk receptor immunoreactivity in individual tumour types is described below.

3.1 *Astrocytoma (grade I and II)*

These were low-grade tumours of the temporal cortex. In grade I protoplasmic astrocytoma, there were characteristic microcystic changes of the tissue, with swollen cytoplasm and moderate cellularity. No mitoses were seen. There was more cellularity in grade II tumours (figure 1d) with little endothelial proliferation. In the surrounding peritumoral tissue, cellularity (neuronal as well as glial) was low (figure 1a, b). GFAP immunoreactivity was present predominantly in astrocytic processes in normal tissues, whereas within the tumour, immunoreactivity was seen only in glial cell bodies (not shown).

In both grades of astrocytomas, there was high expression of Trk A and Trk B receptors (figure 1e, f; grade II astrocytoma, table 2) in glial cells. In grade II forms, the endothelium of tumour capillaries showed conspicuous immunoreactivity (figure 1e, f). This immunoreactivity was considered to be positive, since parallel sections treated with DAB alone (the DAB reaction for peroxidase) revealed no staining in tumour vasculature.

Table 2. Intensity of Trk receptor immunoreactivity in tumour samples examined.

	Intensity	
Tumour type	Trk A	Trk B
Pilocytic astrocytoma I	4^+	4^+
Pilocytic astrocytoma II	4^+	4^+
Protoplasmic astrocytoma I	3^+	3^+
Protoplasmic astrocytoma II	3^+	4^+
Astrocytoma II	3^+	4^+
Giant cell glioblastoma IV	1^+	1^+
Giant cell glioblastoma IV	1^+	1^+
Glioblastoma multiforme IV	1^+	1^+
Glioblastoma multiforme IV	1^+	1^+
Glioblastoma multiforme IV	1^+	1^+

1^+, Very weak; 2^+, weak; 3^+, moderate; 4^+, strong or high.

3.2 *Pilocytic astrocytoma (grade I and II)*

The grade I pilocytic astrocytoma was a tumour (figure 2a) from the cerebellar vermis, the grade II form was from the cortex near the third ventricle. In both cases, there was moderate cellularity, and some endothelial proliferation was present in the grade II tumour. GFAP immunoreactivity revealed the presence of abundant fibrils and elongated cells within the tumours (figure 2b; grade I). In both tumours, Trk A and B expression was the strongest amongst all tumour types examined (figure 2c, d; grade I, table 2). Immunoreactivity for both receptors was widespread in the majority of the tumour cells, while only Trk A was strongly expressed in the tumour vessels in grade II forms (figure 2c). No staining was observed in the capillaries of adjacent peritumoral brain tissue.

3.3 *Giant cell glioblastoma (WHO grade IV)*

In this highly malignant tumour type, there were frequent mitoses, some giant multinucleated cells with abundant cytoplasm and many small tumour cells (figure 3a). In the adjacent peritumoral 'normal tissues', GFAP immunoreactivity was present in many astrocytes and their processes (figure 3b). Within the tumour, fewer GFAP-positive glial cells were seen (figure 3c), suggesting the undifferentiated nature of the glioma. Immunoreactivity for both Trk receptors was low in this tumour. The tumour cells were only occasionally and rather faintly stained (figure 3d, e; table 2).

3.4 *Glioblastoma multiforme (WHO grade IV)*

In this highly malignant tumour form, there was high cellularity and the cells were pleiomorphic. Necrosis was

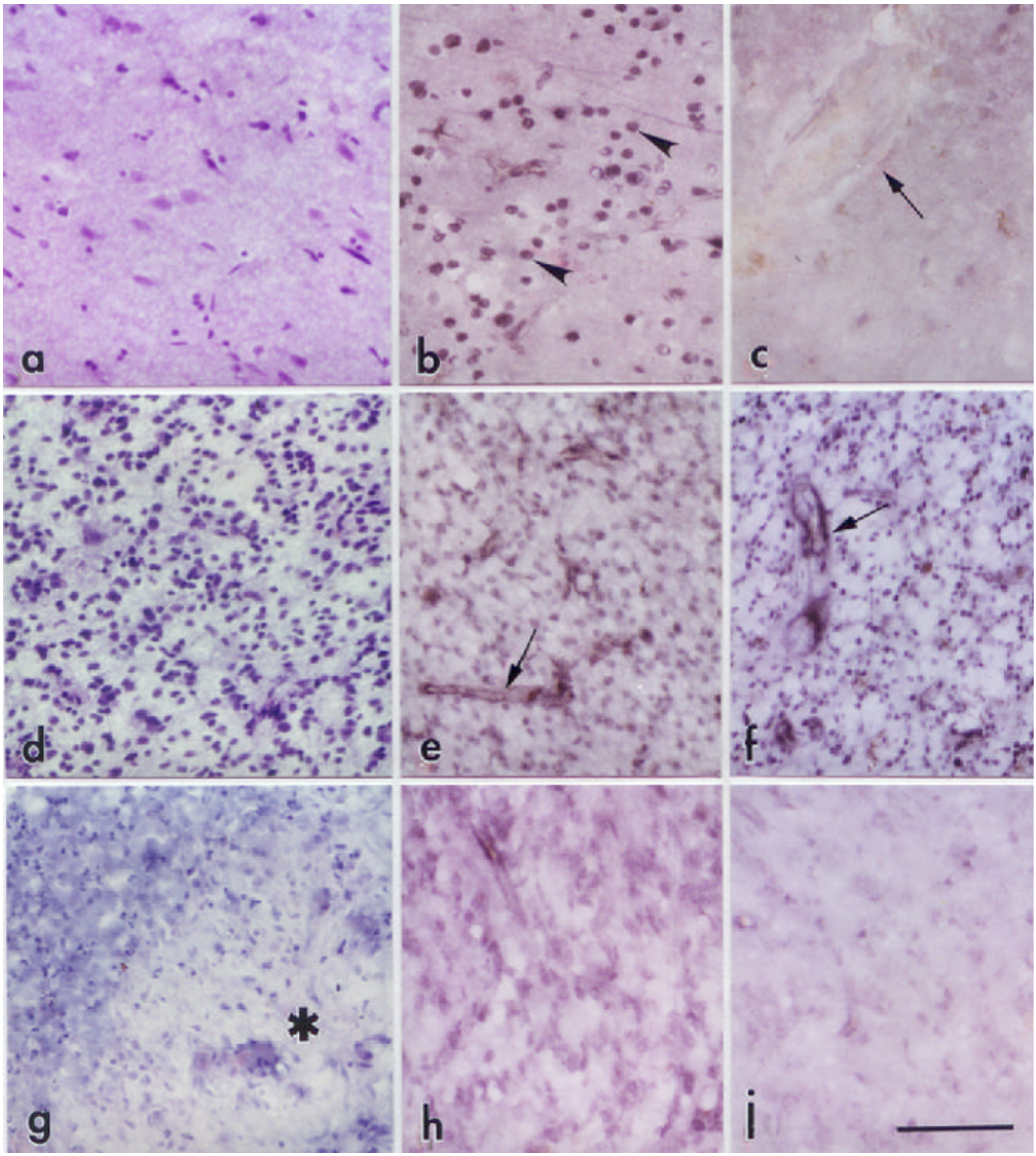

Figure 1. Trk receptor immunoreactivity in different samples of astrocytomas and adjacent peritumoral 'normal tissues'. (**a–c**) From peritumoral tissue adjacent to astrocytoma II, case No. 5: (**a**) H & E stained, showing few cellular elements; (**b**, **c**) showing Trk A and Trk B immunoreactivity, respectively. Neuronal nuclei are immunopositive (arrowheads in **b**); the arrow in (**c**) indicates a blood vessel without staining; (**d–f**) From astrocytoma II, case No. 5: (**d**) H & E stained, showing high cellularity in the tumour tissue; (**e**, **f**) showing Trk A and Trk B immunoreactivity, respectively in this glioma. The arrows indicate staining in the capillary endothelium. (**g–i**) From glioblastoma multiforme (grade IV, case No. 8): (**g**) H & E-stained tumour tissue showing necrosis with lymphocytic infiltration (asterisk) and moderate cellularity; (**h**, **i**) showing Trk A and Trk B immunoreactivity, respectively. In both cases staining intensity is weak (1^+, table 2). Scale bar, 50 μm.

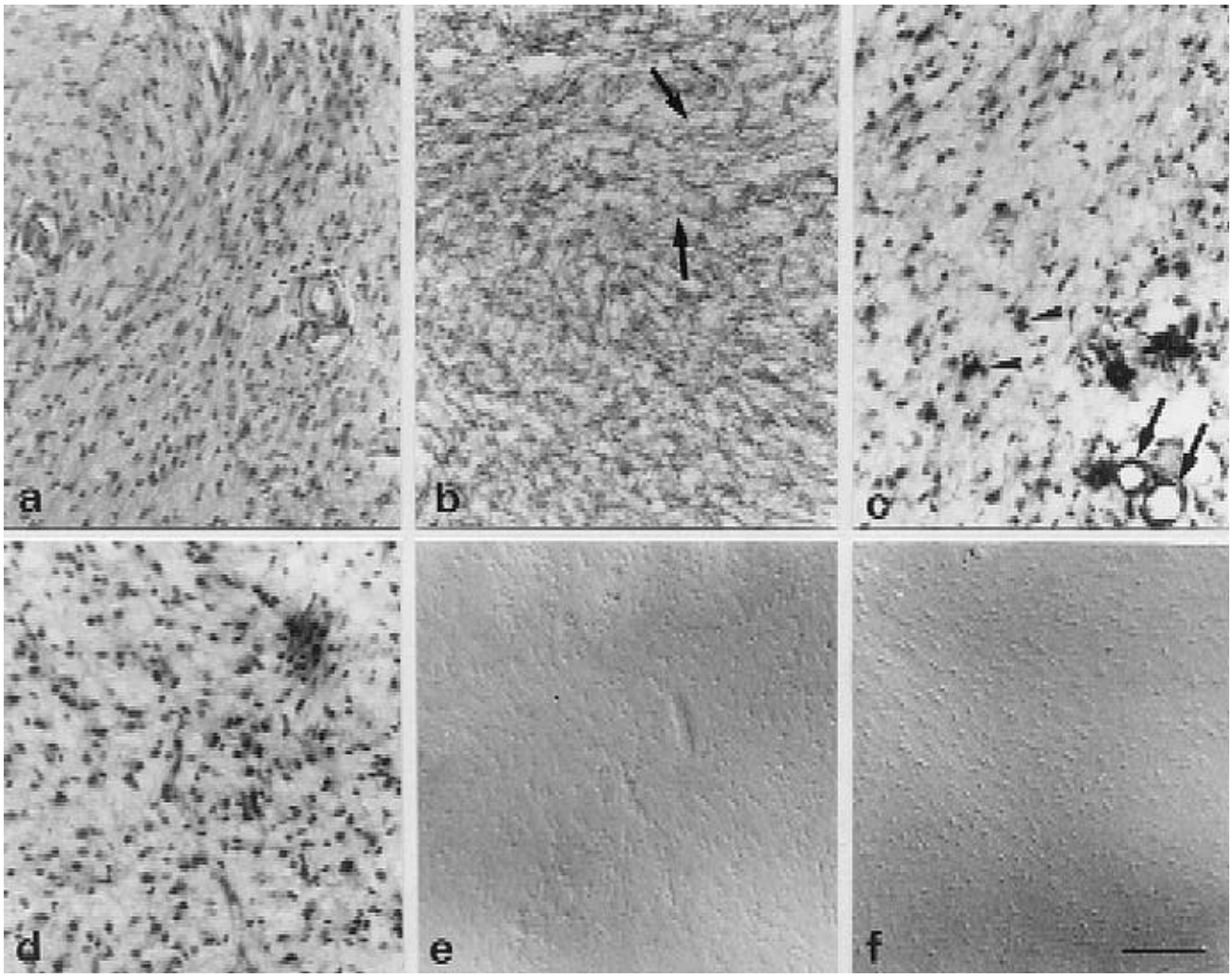

Figure 2. Pilocytic astrocytoma, case No. 1. (**a**) H & E-stained tumour showing moderate cellularity. (**b**) GFAP immunoreactivity in the adjacent tumour section. The abundant fibrils (arrows) are characteristic to this tumour. (**c**, **d**) Showing Trk A and Trk B immunoreactivity in the tumour cells respectively. The tumour vessels are strongly positive for Trk A (**c**, arrows). (**e**, **f**) Control sections treated with primary antibodies preabsorbed with respective control peptides, showing no staining for Trk A (**e**) and Trk B (**f**). Scale bar, 50 μm.

evident over a large area within the tumour (figure 1g) and there was marked proliferation of blood vessels. As in the giant cell glioblastoma, the cellular elements of this tumour showed very weak immunoreactivity (figure 1h, i). Capillary endothelium was also faintly stained. Control sections treated with the primary antisera preabsorbed with respective control peptides did not show any staining (figure 2e, f). Also omission of the primary antisera did not reveal any staining in the tumour sections (figure 3f).

4. Discussion

Previous reports have shown that neurotrophins as well as their cognate receptors are expressed predominantly in the developing nervous system. Physiologically speaking, a given neuron does not express a growth-factor receptor until it is ready for interaction with its ligand (Yan and Johnson 1998). However, information about the distribution and possible role of neurotrophin receptors on glia is somewhat limited (Althaus *et al* 1992; Jelsma *et al* 1993; Barres *et al* 1994; Ikeda and Puro 1994). A truncated form of Trk B is reported to be expressed in glia of white matter (e.g. optic nerve), wherein the full-length form of Trk B, being involved in cell proliferation and/or differentiation, does not express (Klein *et al* 1990; Merlio *et al* 1992; Jelsma *et al* 1993). Under normal physiological conditions, the glial cells do not express NGF receptors; however, in response to injury, the oligoden-

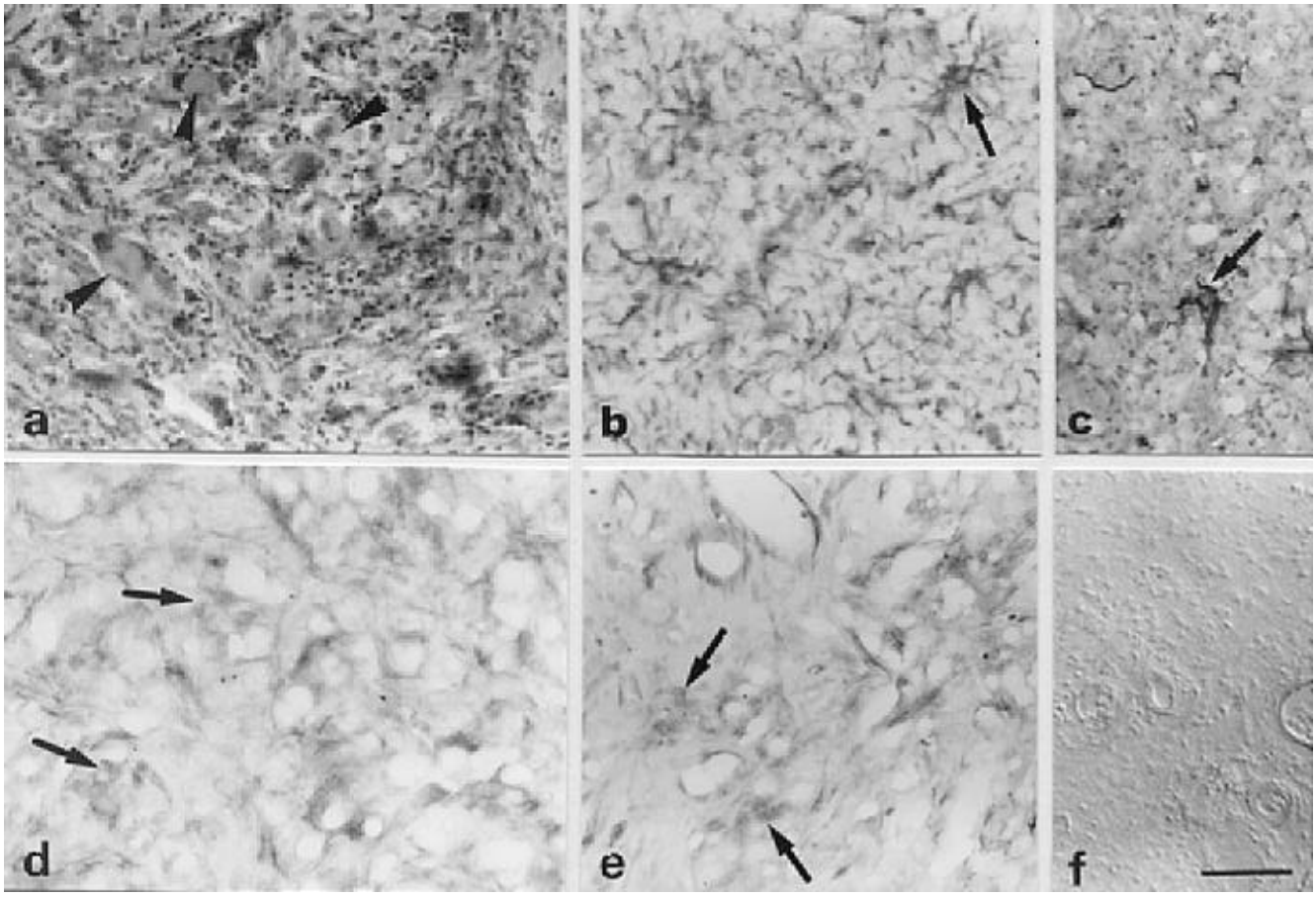

Figure 3. Giant cell glioblastoma, grade IV, case No. 6. (**a**) H & E-stained tumour showing small tumour cells and some giant cells (arrowheads). (**b**) Adjacent peritumoral tissue, showing GFAP immunoreactivity in normal astrocytic processes (arrow). (**c**) From the tumour tissue. GFAP immunoreactivity is present in few glial cells (arrow) and their processes. (**d**, **e**) Showing weak staining (arrows) for Trk A and Trk B, in this malignant tumour respectively (see table 2). (**f**) Control tumour section (case No. 5) processed without primary antiserum (Trk A) treatment. No staining is present in tumour cells or capillaries. Scale bar, 50 μm.

drocytes express NGF receptor (Althaus *et al* 1992). Hunton *et al* (1992) showed the presence of mRNAs for NGF receptors (p75 and Trk A) and Trk B in rat C_6 glioma cells. Here we report the predominant expression of Trk A and Trk B in adult human astrocytic tumours, especially in the benign state. It is possible that both receptors, by interacting with their neurotrophin ligands, may mediate glial proliferation in early astrocytoma.

In childhood neuroblastoma, there are reports on the expression of Trk A as well as p75-neurotrophin receptor (Donovan *et al* 1993; Nakagawara *et al* 1993; Dominici *et al* 1997). Trk A expression is high in the majority of neuroblastoma cases studied, especially in non-advanced stages (Borrelo *et al* 1993; Nakagawara *et al* 1993; Dominici *et al* 1997), and this has been associated with a favourable outcome (Kogner *et al* 1993; Nakagawara *et al* 1993). On the other hand, in aggressive, high-risk neuroblastoma there is a lack of Trk A expression, though Trk B expression therein has been reported (Brodeur *et al* 1997). The expression of Trk B in neuroblastoma is most probably related to tumour behaviour and outcome (Nakagawara *et al* 1994; Brodeur 1995; Brodeur *et al* 1997).

In pediatric medulloblastoma, there are reports suggesting involvement of several neurotrophins and their cognate receptors in tumour pathogenesis (Keles *et al* 1993; Washiyama *et al* 1996). However, unlike Trk A, which is expressed predominantly in neuroblastoma, as already mentioned, Trk B (and also Trk C) expression in medulloblastoma appears to be characteristic (Washiyama *et al* 1996). The present study shows that Trk A as well as Trk B is expressed at high levels in low grade astrocytoma, as also reported by Wang *et al* (1998). We believe that this overexpression might contribute to the progression of early neoplasms. On the contrary, in advanced, highly malignant forms of these tumours, there is an apparent

downregulation of both Trk receptors. However, this was not reported by Wang *et al* (1998). Our findings bear similarity to the cases of aggressive neuroblastoma studied by Brodeur (1995), in which there was a reported lack of Trk A and Trk C expression. Thus there appears to be a strong correlation between expression of Trk receptors and malignancy of tumours (neuroblastoma and astrocytoma); the more malignant the tumour, the lower the level of Trk expression. The reason for this apparent receptor downregulation at advanced stages of the tumours is not clear. However, it does appear that the tumour cells become less responsive to the available neurotrophins during malignancy, to lead to growth factor-independent proliferation.

Trk receptors are expressed also in non-neuronal tissues too, e.g. kidney (Ernfors *et al* 1991; Durbeej *et al* 1993) and in pediatric renal neoplasms, as in Wilms' Tumour (Donovan *et al* 1994). In our study, an interesting finding hitherto unknown and not reported by Wang *et al* (1998) is the localization of Trk immunoreactivity in capillaries of grade II tumours. It is possible that Trk receptors may mediate endothelial proliferation in early stages of the astrocytoma, but seemingly play no role in this process in the advanced malignant forms.

In summary, this study reports the expression of Trk A and Trk B in adult human astrocytoma and glioblastoma, suggesting a role for these receptors in glial and endothelial proliferation, especially in early stages of the neoplasms.

Acknowledgements

TCN received a Senior Research Associateship from the Council of Scientific and Industrial Research, New Delhi.

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MS received 15 July 2002; accepted 21 January 2003

Corresponding editor: PAUL T VAN DER SAAG