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Glial cell line-derived neurotrophic factor (GDNF) and its receptor (GFR-α1) are strongly expressed in human gliomas

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Abstract Glial cell line-derived neurotrophic factor (GDNF), a sequence-related factor of the transforming growth factor-β family, has been identified as a potent neurotrophic factor for a variety of neuronal cell populations. At present, it is still unknown whether human gliomas in vivo are also capable of producing GDNF. We studied the expression of GDNF in 14 human glioblastomas, 1 gliosarcoma and 5 astrocytomas. Using an enzyme-linked immunosorbent assay, the amount of GDNF was quantified in human gliomas and compared to GDNF-expression in C6 glioma cells, mouse fibroblasts and normal human and rat brain. Mean concentration of GDNF in gliomas was 937 ± 140 pg GDNF/g tissue (n = 20). C6 cells revealed the highest expression levels of 2,837 ± 813 pg/g, whereas mouse 3T3 fibroblasts showed no detectable GDNF protein. Mean GDNF tissue levels in normal human and rat brain were significantly lower. Using reverse transcriptase-polymerase chain reaction, GDNF mRNA was detected in human gliomas and in rat C6 cells. Immunohistochemistry revealed strong GDNF- and GDNF receptor-α1-expressing tumor cells in human glioma tissue. These results show that glial tumors, even in the most dedifferentiated form of glioblastoma, express GDNF at concentrations up to five times higher compared to normal human brain. This overexpression of GDNF may be of biological relevance for proliferation of glial tumors in humans.

Key words Growth factors · Glial cell line-derived neurotrophic factor · Brain tumors · Glioma

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Introduction

Approximately 40% of all CNS tumors are primary tumors derived from glial cells such as astrocytes, oligodendrocytes and ependymal cells. Astrocytomas comprise 60–70% of glial cell tumors [6]. High grade gliomas display malignant cellular features such as extreme hypercellularity, pleomorphism, mitotic figures, multinucleation, and bizarre giant cell forms [14, 20, 30, 31]. Neurotrophic growth factors have been found to be important mediators of proliferation, differentiation and survival in the normal brain [7, 8, 10], but their role in brain tumors remains mostly unclear. Various growth factors have been identified as being crucial in the biology of gliomas, exerting their effects on cellular proliferation and differentiation by activating intracellular signaling mechanisms. At present, transforming growth factor-α [29] and-β [31], acidic and basic fibroblast growth factor [35], platelet-derived growth factor [21] and vascular endothelial growth factor [25] are thought to represent the most relevant growth factors for glial tumors. Other studies have focused on nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 in medulloblastomas and other primitive neuroectodermal tumors of the pediatric CNS [42]. These studies reported that both the neurotrophins and their cognate receptor proteins [1, 41] are expressed in gliomas and may influence the induction or progression of these neoplasms [17].

Glial cell line-derived neurotrophic factor (GDNF) was discovered in conditioned medium of the B49 rat glioma cell line [18]. GDNF is synthesized as a precursor and secreted as a mature protein of 134 amino acids. It contains the 7 conserved and typically spaced cysteine residues found in all members of the TGF-β superfamily

but shares less than 20% identity with any family member [18]. GDNF has been identified as a potent neurotrophic factor promoting survival and differentiation of a wide range of neuronal cell populations, such as dopaminergic, noradrenergic or cholinergic neurons, Purkinje cerebellar neurons, facial nerve and spinal cord motoneurons, and striatal and hippocampal neurons [16, 24, 38]. The expression of GDNF is not only restricted to neurons [13, 26, 32, 34]; it is also expressed in rat and human astrocytes and glioma-derived cell lines [22, 28, 33, 39, 40]. At present, however, it is still unknown whether human astrogliomas can produce and secrete GDNF during the process of malignant transformation.

To determine whether GDNF could be relevant for the biology of glial tumors in humans we studied the expression of GDNF in various human gliomas at the protein and mRNA level. Immunohistochemistry was used to analyze the cellular distribution of GDNF and its receptor GFR-αl. Reverse transcriptase (RT)-PCR was used to detect GDNF mRNA in selected tumor tissue samples. By means of ELISA the amount of GDNF was quantified in human gliomas and in normal human brain and compared to GDNF expression in C6 glioma cells and normal rat brain.

Materials and methods

Tissue samples

Twenty glial neoplasms, resected between 1996 and 1999, were included in this study and comprised 14 glioblastomas and 1 gliosarcoma (WHO grade IV), and 5 astrocytomas (WHO grades I–III). Tumors were diagnosed according to the WHO classification of brain tumors [14]. The freshly obtained tumor samples were cut into two halves, one was fixed in formalin and embedded in paraffin for histological diagnosis; the other was frozen in a CO_2 stream and then stored at –70 °C. Only in 4 of the 20 tumor samples enough tissue could be obtained to perform ELISA, immunohistochemistry and RT-PCR in parallel. Due to the limited sample size, 10 gliomas were analyzed by ELISA and immunohistochemistry only, and 4 gliomas were studied by ELISA alone. The details on the methods applied are given in Table 1.

Normal human brain tissue was obtained from autopsy within 10 h of the patient's death, dissected and processed as described above. Adult Sprague Dawley rats were killed and the following brain areas were dissected and frozen: parietal cortex, striatum, ventral mesencephalon and cerebellum. Rat C6 glioma cells and 3T3 mouse fibroblasts were cultured in DMEM medium containing 10% fetal calf serum in a large petri dish (10-cm diameter) until 80% confluency, trypsinized, collected and frozen. All brain tissues and cell pellets were weighed before they were frozen at –70 °C.

Tissue extraction

GDNF protein extraction was performed by acidic extraction as described previously [23]. Tissue samples and cultured cells were thawed and sonicated five times on ice in 1 × sample buffer (Promega) adjusted to pH 2.5. Samples were incubated for 15 min at room temperature and then neutralized (pH 7.5). After centrifu-

Table 1 GDNF in human gliomas. GDNF protein concentrations were measured by ELISA. Tumors were histologically diagnosed according to the WHO classification. The values are given as pg GDNF/g tissue, and were obtained from three different dilutions (undiluted, 1 : 10 and 1 : 100 diluted samples) with duplicate measurements, and values were averaged. RT-PCR revealed a clear single band in the agarose-gel (+). Immunohistochemistry for GDNF (GDNF-LI)- or GFR-α1 (GFR-α1-LI)-like immunoreactivity showed strong (++) or weak (+) labeling. Due to the limited sample material not all tumor samples could be analyzed by all methods (*GDNF* glial cell line-derived neurotrophic factor, *GFR-α1* GDNF receptor-α1)

Case no.	Sex	Age (years)	Histology (WHO grade)	GDNF (pg/1 g tissue)	RT-PCR	GDNF-LI	GFR-α1-LI
1	M	39	Gliosarcoma (IV)	882		+	+
2	F	38	Glioblastoma (IV)	2,755		++	
3	F	65	Glioblastoma (IV)	2,146	+	++	++
4	M	68	Glioblastoma (IV)	1,042			
5	M	45	Glioblastoma (IV)	764		+	
6	M	61	Glioblastoma (IV)	701		++	++
7	F	55	Glioblastoma (IV)	1,664	+	++	++
8	F	28	Gemistocytic astrocytoma (II)	1,040		++	++
9	F	37	Fibrillary astroyctoma (II)	640		+	++
10	F	30	Fibrillary astrocytoma (II)	963			
11	F	37	Glioblastoma (IV)	707	+	+	+
12	M	53	Glioblastoma (IV)	248		+	+
13	M	46	Glioblastoma (IV)	411		+	+
14	M	52	Glioblastoma (IV)	333	+	++	++
15	F	74	Glioblastoma (IV)	1,022			
16	F	30	Anaplastic astrocytoma (III)	971		++	++
17	F	68	Glioblastoma (IV)	826			
18	M	52	Glioblastoma (IV)	918		++	
19	M	19	Subependymal astrocytoma (I)	625			
20	M	65	Glioblastoma (IV)	92			

gation at 13,000 *g* for 5 min, the supernatants were collected and used for ELISA.

ELISA

GDNF concentrations in lysates of tissues and cells were determined by ELISA (Promega) as described previously [39]. The antibody used cross-reacts with GDNF of various mammalian species, but does not cross-react with TGFs, BDNF or NGF. Briefly, 96-well ELISA plates were coated with an anti-GDNF monoclonal antibody diluted in carbonate coating buffer (pH 8.2) and incubated overnight at 4 °C. Plates were blocked for 1 h at room temperature with 1 × blocking buffer (Promega, 200 µl/well). GDNF standards ranging from 0 to 100 pg/100 µl were prepared using recombinant GDNF (Promega) and applied to the wells. Tissue extracts (see above) were added to the wells (100 µl) undiluted, or diluted 1 : 10 and 1 : 100. All samples were done in duplicate. Samples and standards were incubated at room temperature for 6 h on a shaker and then washed five times in washing buffer. The plate was then incubated with chicken anti-human GDNF polyclonal antibody overnight at 4 °C. After plates were again washed five times, horseradish peroxidase-conjugated anti-chicken antibody (1 : 5,000) was added to the plates and incubated at room temperature for 2 h on a shaker. Plates were again washed, and the enzyme substrate (TMB and peroxidase, Promega) added and incubated for 15 min at room temperature. The enzyme reaction was stopped by adding 100 µl 1 M phosphoric acid per well, and the absorbance was measured at 450 nm. Samples were calculated from the standard curve in the linear range. Tissue concentrations were calculated from the weight of the tissue/cells. All samples were analyzed by one-way ANOVA, and compared with a Fisher PLSD posthoc test, when the ANOVA revealed a significance of $P < 0.05$.

RT-PCR

mRNA was isolated from glioblastoma tissue of four patients (Table 1) and from C6 glioma cells. Briefly, tissue/cells were homogenized in a solution containing 4 M guanidinium isothiocyanate, incubated in phenol/chloroform/sodium acetate for 15 min at 4 °C, centrifuged at 13,000 *g* for 20 min (4 °C) and precipitated for 3 h at –70 °C with 1 vol isopropanol. Each sample was centrifuged at 13,000 *g* for 20 min at 4 °C, the pellet dissolved in the RNA homogenization solution and again precipitated with isopropanol overnight at –70 °C. After centrifugation (10,000 *g*, 4 °C) the pellet was washed in 70% ethanol/sodium acetate, again centrifuged dried and resuspended in distilled water. RNA was measured at 260 and 280 nm in a photometer. Total RNA (2 µg) was incubated in a reaction buffer containing 100 µg/ml antisense GDNF primer (see below), 0.5 mM dNTPs, RNase inhibitor and 40 U MMULV reverse transcriptase (Boehringer Mannheim, Vienna) for 10 min at 25 °C and for 90 min at 37 °C and then heated for 10 min at 80 °C. The cDNA was then subjected to PCR amplification. Reactions were carried out in 0.2-ml tubes. Reagents were assembled in a final volume of 25 µl containing 3 µl of the RT reaction, 1 µl 100 µM sense (5′-GCT AGG TAC CAT GAA GTT ATG GGA TGT CG-3′) and antisense (5′-GCA TCT CGA GTC AGA TACATC CAC ACC G-3′) GDNF primers, 1 µl 10 mM dNTPs, and 1 U Taq DNA polymerase (Boehringer Mannheim). Samples were initially denatured for 3 min at 97 °C before addition of the polymerase and overlayed with mineral oil. Thermocycling parameters were 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, which was repeated for 35 cycles, and stopped with a 5 min incubation at 72 °C. Aliquots (20 µl) of the PCR reaction were run on a 1% agarose-ethidium-bromide gel, visualized under UV and photographed using a Polaroid camera.

Immunohistochemistry

Immunohistochemistry using the avidin-biotin technique was performed as described previously [9]. Frozen tissue was sectioned (40-µm-thick sections) in a cryostat (Leitz). Tissue sections were air dried, postfixed for 10 min with 4% paraformaldehyde/PBS, rinsed three times in 0.05 M TRIS-buffered saline (TBS) for 10 min at room temperature and incubated with primary polyclonal antibodies against glial fibrillary acidic protein (GFAP; 1 : 200, Sigma), GDNF (1 : 200, Promega) and GFR-α1 (1 : 500, Serotec) in a humidity chamber overnight at room temperature. The sections were washed and incubated with secondary anti-rabbit (GFAP, GFR-α1) or anti-chicken (GDNF, Promega) biotinylated antibodies (1 : 200) for 1 h at room temperature. After washing, sections were incubated in Vectastain reagent (Vectastain) for 30 min and washed in 50 mM TRIS-HCl pH 7.6. The signal was detected using 0.5 mg/ml 3,3′-diaminobenzidine (DAB) as chromogen. The sections were dehydrated and mounted with Entellan. Omission of the primary antibody was used as a negative control.

Results

GDNF protein concentration

The ELISA standard curve was linear between 1.5 and 100 pg/100 µl corresponding to 0.05 and 0.55 absorbance units at 450 nm (not shown). The ELISA analysis revealed high GDNF tissue levels in most human gliomas (Fig. 1, Table 1). Similarly, rat C6 glioma cells contained very high amounts of GDNF protein (Fig. 1). Human postmortem tissue of normal brain (white matter as well as gray matter) and different rat brain tissues contained low levels of GDNF protein (Fig. 1). In contrast, mouse 3T3 fibroblasts did not contain GDNF protein at all (Fig. 1).

RT-PCR of GDNF

GDNF-specific RT-PCR of rat C6 glioma cells resulted in a strong PCR product of approximately 700 bp (Fig. 2) in four analyzed tumor samples (Table 1). A slightly weaker

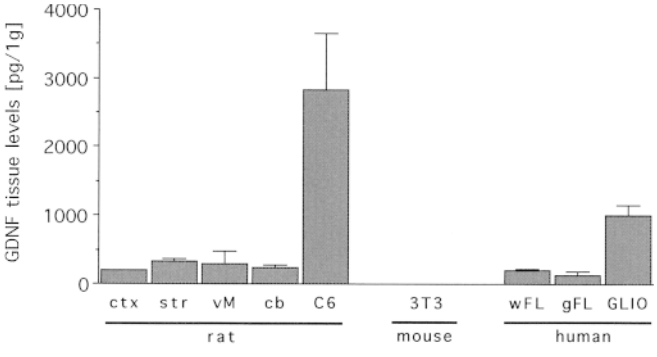

Fig. 1 GDNF concentrations in various rat, mouse and human brain tissues, gliomas and cell lines, as determined by ELISA. GDNF was measured in rat cortex *(ctx)*, striatum *(str)*, ventral mesencephalon *(vM)*, cerebellum *(cb)*, mouse 3T3 fibroblasts *(3T3)* and in rat C6 glioma cells *(C6)*. In human tissue GDNF was measured in postmortem white matter frontal lobe *(wFL)* and gray matter frontal lobe *(gFL)* as well as in human gliomas *(GLIO)*. Values are given as mean ± SEM in pg GDNF/g tissue (n = 3–4 for rat and mouse tissue; n = 5 for postmortem tissue; and n = 20 for gliomas) (*GDNF* glial cell line-derived neurotrophic factor)

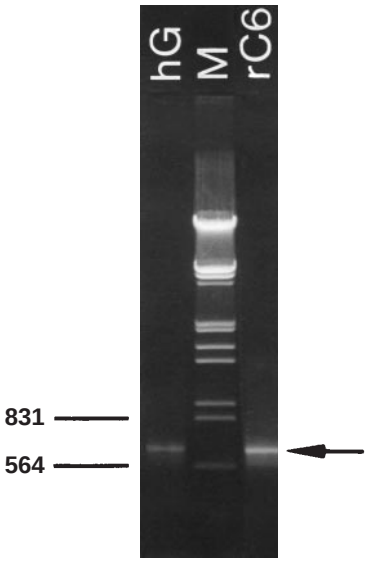

Fig. 2 GDNF mRNA in human gliomas and rat C6 glioma cells. GDNF from human gliomas (hG) and rat C6 glioma cells (rC6) was subjected to RT-PCR. Total RNA was isolated, reverse transcribed, subjected to PCR and analyzed on a 1% agarose-ethidiumbromide gel. The size of the PCR products was verified using a DNA marker *(M)*. The base pair length is given on the *left*. The *arrow* points to the GDNF PCR product

smaller band was visible (Fig. 2). RT-PCR of human gliomas revealed a PCR product of similar size (Fig. 2). In situ hybridization was below the detection limit to measure GDNF mRNA at the cellular level (data not shown).

GDNF and GFR-α1 immunohistochemistry

GFAP-immunohistochemistry showed strong labeling of many tumor cells (data not shown). Immunohistochemistry for GDNF revealed strong cytoplasmic staining of tumor cells (Fig. 3). The same pattern of staining was found with the immunohistochemical analysis for the GDNF receptor GFR-α1 (Fig. 3). The intensity of staining varied from weak (+) to strong (++) as given in Table 1.

Discussion

The findings reported in this study show that the novel growth factor GDNF is highly expressed not only in rat glioma cells in vitro but also in human gliomas in vivo.

The expression of growth factors in gliomas has attracted a lot of interest because of their significance for regulation of growth and differentiation, thereby leading to new therapeutic interventions. Several studies reported that growth factors and their cognate receptors influence the induction or progression as well as the differentiation of neoplasms [5, 17]. GDNF, a sequence-related member to the TGF-β superfamily, is expressed in primary cultured astrocytes of rats [28] and humans [22] and in activated macrophages and microglia [3], possibly playing a potent role in differentiation or proliferation. Since GDNF expression has been found in high concentrations in rodent and human-derived glioma cell lines such as B49 [19], C6 [39] or U-87MG [40] cells, a role in malignant tumor transformation has been suggested. GDNF was also found in the SK-N-AS neuroblastoma cell line [40] but not in a pheochromocytoma cell line [33].

Several research groups reported that GDNF synthesis and/or release can be regulated by numerous factors, signaling through multiple and diverse secondary messenger systems [33, 39, 40]. Ho et al. [12] demonstrated that striatal astrocytes express GDNF after exposure to the glutamate agonists *N*-methyl-L-aspartate or kainic acid, indicating a role as a survival factor. The expression of GDNF can also be induced by another important glial growth factor, fibroblast growth factor-2 (bFGF) [33], possibly indicating additive or synergistic effects.

Our data show that glial tumors, even in the most dedifferentiated forms of glioblastoma and gliosarcoma, express GDNF at concentrations up to five times higher compared to normal human brain. The expression of GDNF protein was demonstrated by a sensitive quantitative ELISA as well as at the cellular level using immunohistochemistry. RT-PCR confirmed the expression of GDNF mRNA showing a major transcript of approximately 700 bp and a weak smaller transcript of approximately 620 bp, which is in line with RT-PCR results of previous studies [33, 39].

GDNF utilizes a novel multireceptor signaling system. GDNF binds to a high-affinity receptor, termed GDNF receptor-α1 (GFR-α1), which is anchored to the plasma membrane via a glycosyl-phosphatidyl-inositol protein [4, 15, 36]. The signaling receptor is the receptor tyrosine kinase RET [27, 37, 43]. Recently, sequence-related members have been cloned (neurturin, persephin and artemin), and it has been demonstrated that all of them use the same receptor RET; however, neurturin prefers a GFR-α2, persephin a GFR-α4 and artemin a GFR-α3 receptor [2]. Using immunohistochemistry we were able to detect the GFR-α1 in human gliomas. Our data confirm that not only the GDNF protein but also its receptor is present in the malignant tumors.

Aguado et al. [1] as well as Wang et al. [41] showed that human astrocytic gliomas and reactive astrocytes express the TrkA, B and C receptors, indicating a role for neurotrophins in neoplastic proliferation. Since astroglial cells are capable of producing NGF and TrkA, it was suggested that NGF may function as an autocrine or paracrine factor for reactive and neoplastic glial cells [1]. Since our data demonstrate that GDNF as well as its receptor GFR-α1 are found in human gliomas, it seems reasonable to speculate that GDNF may also act as an autocrine or paracrine factor for gliomas in vivo. This overexpression of GDNF in human gliomas may be of biological relevance for growth and differentiation of these glial tumors. Recently, Franke et al. [11] suggested that CNS glial cells are targets for GDNF as well as for neurturin, acting possibly via such an autocrine/paracrine mechanism, at least in the rat.

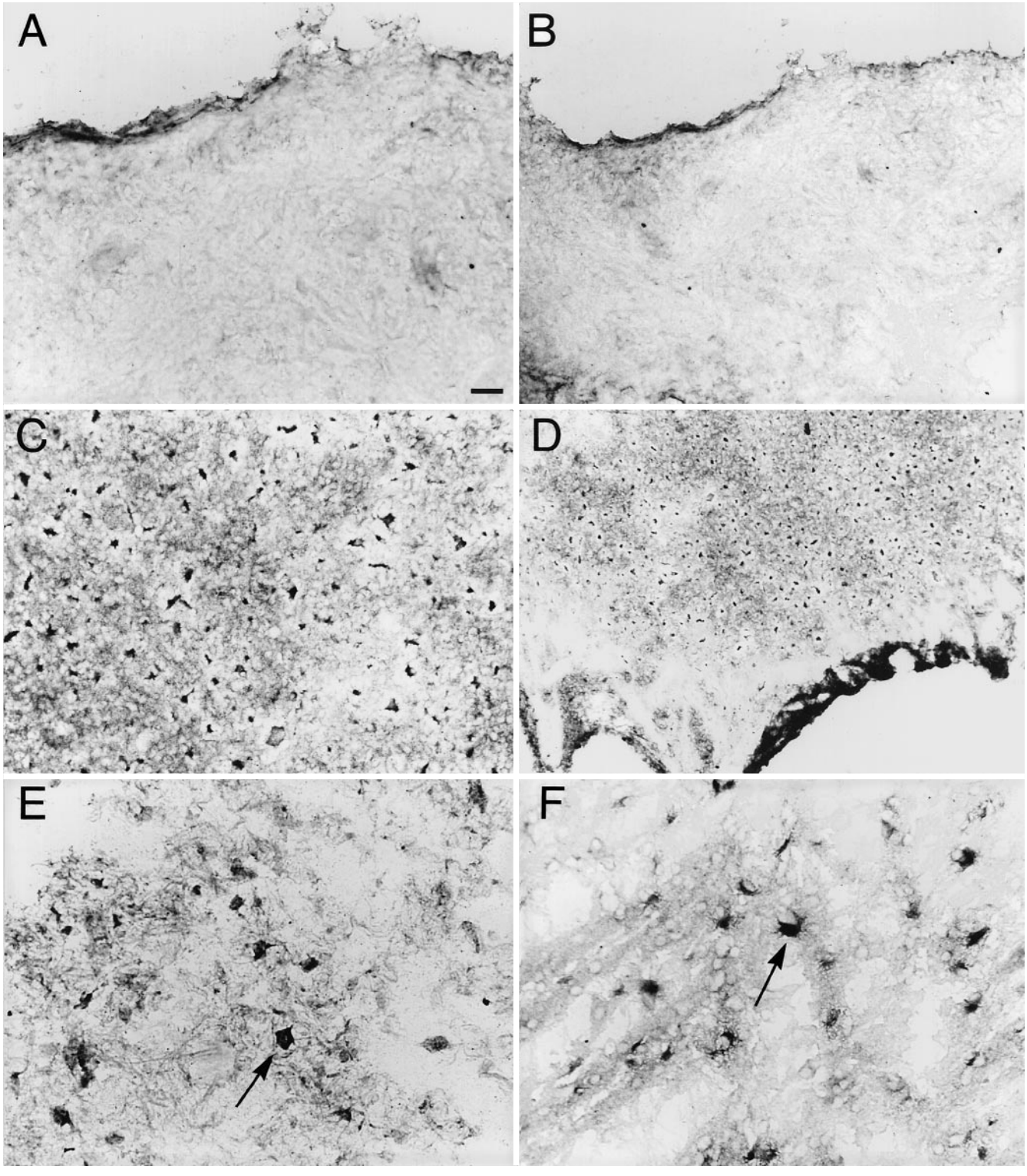

Fig. 3 A–F Immunohistochemistry for GDNF and GFR-αl in human glioblastomas. **A**, **B** Controls where the GDNF (**A**) or the GFR-α1 (**B**) primary antibodies were omitted. **C**–**F** Immunohistochemical staining with the GDNF (**C**, **E**) and the GFR-α1 (**D**, **F**) antibodies. The *arrows* in **E** and **F** point to strongly stained glial tumor cells (*GFR-α1* GDNF receptor-αl). *Bar* **A**, **C** 190 μm; **B**, **D** 500 μm; **E**, **F** 95 μm

In conclusion, our data show that GDNF and its receptor GFR-α1 are highly expressed in human gliomas, which may indicate a role of GDNF for proliferation of human gliomas. To further determine the biological significance of GDNF overexpression in human gliomas, additional studies are necessary by blocking GDNF expression and investigating its effects on growth behavior and differentiation.

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