1997-02-01

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TrkA Expression Decreases the *in Vivo* Aggressiveness of C6 Glioma Cells[1]

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ABSTRACT

We stably expressed the nerve growth factor receptor trkA or a truncated trkA lacking the kinase domain (trkAΔ) in a highly tumorigenic rat glioma cell line, C6. Survival of rats with large intrastriatal inocula of C6[trkA] cells was significantly longer than for rats bearing C6 or C6[trkAΔ] cells. Histological studies revealed that C6[trkA] cells were much less invasive than C6 or C6[trkAΔ] cells and had a greater rate of apoptosis. There was no apparent induction of differentiation of C6 cells by trkA. Therefore, unlike what is observed in neuroblastomas, trkA decreases tumorigenicity by modulating invasiveness and tumor cell death independent of inducing differentiation. This novel mechanism suggests a new therapeutic strategy for malignant gliomas.

INTRODUCTION

Malignant gliomas are highly invasive, rapidly proliferating tumors that even with the most aggressive conventional treatments are still associated with short survivals. Not all glial tumors are malignant, however, and in certain instances these tumors can remain indolent for years or be curable with surgery alone (1–5). Although studies address the molecular basis of glioma malignancy, little attention has been directed at the question of why other gliomas are benign.

In contrast, the benign character of some other neural tumors is better understood. Patients with neuroblastomas expressing NGF[3] receptor trkA have a much better prognosis than those lacking trkA expression (6, 7). Likewise, expression of neurotrophin-3 receptor, trkC, by medulloblastomas is associated with a favorable outcome (8). Consistent with these clinical findings, overexpression of trkA in neuroblastoma cell lines enhances NGF-induced differentiation both *in vitro* and *in vivo* (9–11). Malignant gliomas do not express trkA (12–14), but trkA expression can be induced with expression vectors.

We chose to test the effects of trkA expression on the C6 rat glioma cell line because it has a well-characterized neuroglial phenotype and because it rapidly and extensively invades rat brain postimplantation, making it a useful model for human brain tumors (15–17). We found that trkA-expressing C6 cells are not induced to differentiate but are less invasive and have a higher rate of apoptosis *in vivo*. Furthermore, rats bearing these cells survive significantly longer than those bearing parental ones, demonstrating that trkA expression can decrease tumor aggressiveness by gliomas, apparently by a novel mechanism.

MATERIALS AND METHODS

Establishment of Cell Transfectants. C6 rat glioma cells (ATCC cell lines and hybridomas) were transfected with expression vectors encoding

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either a full-length rat trkA receptor (pLEN trkA, donated by Dr. A. Ullrich, Germany) or a truncated trkA lacking the tyrosine kinase domain. The latter plasmid was constructed by digesting pLEN trkA with EcoRI. The 2.7-kb trkA insert was purified by agarose gel electrophoresis and ligated overnight with an EcoRI-digested TA vector (Invitrogen). TA-trkA was digested with DraIII. Linkers d(5'-CTA GAC TAG TCT AGT TT-3') and d(3'-ACC GAT CTG ATC AGA TC-5') containing a SpeI site and an amber stop codon were phosphorylated and ligated to the DraIII 4.5-kb and 2-kb fragments. These fragments were then ligated to form plasmid TA-trkAΔ. The 2.2-kb EcoRI trkA fragment from TA-trkAΔ was ligated to the EcoRI-digested pLEN vector. The orientation of the insert for plasmid pLEN-trkAΔ was confirmed using HindIII, PvuI, and SpeI. The molecular weight of the trkAΔ protein product was predicted to be 44 kDa less than that of trkA. C6 glioma cells with 30–35 μg of linearized plasmid were electroporated at 960 μFd at 0.45 V with infinite resistance. The cells were then resuspended in 10 ml of serum-supplemented media and incubated for 48 h before selection was initiated in 300 μg/ml G418 (Life Technologies, Inc.).

Flow Cytometry. For flow cytometric analysis, 10^6 cells were incubated at 4°C for 2 h with 10 μg of either TA-1 mab (murine IgG3) or an isotypic IgG3 control mab (Sigma Immunochemicals). The cells were then washed twice in MEM and resuspended in 1 ml of PBS containing 1 μg of FITC-labeled goat anti-mouse antibody (Becton Dickinson). After two additional washes, cells were fixed in 1% paraformaldehyde, and flow cytometric analysis was performed on a FACScan(BDIS). The fluorescence intensity of 10,000 viable cells was measured.

RT-PCR. Poly(A)$^+$ RNA was prepared using the QuickPrep mRNA Purification kit (Pharmacia Biotech). RNA was heat denatured at 95°C for 5 min and then quickly chilled on ice. Reverse transcriptase buffer, deoxynucleotide triphosphates, RNase inhibitor, and oligo(dT) (Perkin-Elmer GeneAmp RNA PCR kit) were mixed with 1 μg of poly(A)$^+$ RNA and the reaction was carried out in a Perkin-Elmer thermal cycler 480 using the RT program (15 min at 42°C followed by 5 min at 99°C and cooling to 4°C). Oligonucleotide primers, buffer, and Taq polymerase were then added and after initial heating of the mixture to 95°C for 2 min, the PCR amplification was carried out for 35 cycles (1 min at 94°C, 1 min at 58°C, and 2 min at 72°C) with a final extension of 10 min at 72°C. PCR products then were analyzed on a 2% agarose gel and stained for 1 h with ethidium bromide. The primer sequences for trkA and sizes of the predicted PCR products were: 5'-CGT CAT GGC TGC TTT TAT GG-3'; 5'-ACT GGC GAG AAG GAG ACA C-3', 75 bp (18). For Western blotting, cells were extracted with NP40-containing buffer. Protein was normalized using the method of Sheffield et al. (19), and trkA was immunoprecipitated with TA-1 mab (ascites, 1:250 dilution; Ref. 20). Western blots were then performed using polyclonal anti-trkA RTA antibody.

MTT Growth Assay. Logarithmically growing cells were trypsinized and 20,000 cells in 500 μl of medium were added to a 24-well plate and then incubated for 2 h at 37°C. NGF was diluted at the desired concentration in 500 μl of media, added to each well, and incubated for 4 days. Experiments were performed in quadruplicate in DMEM/F-12 1:1 serum-free media supplemented with insulin and transferrin. At the end of the incubation period, 100 μl of MTT ($C_{18}H_{16}N_5SBr$; Sigma) solution (5 mg/ml in PBS) were added to each well and incubated for 2 h at 37°C, after which cells were triturated in 1.1 ml of MTT solubilization solvent solution (Sigma). Three hundred μl of this mixture were transferred in duplicate to a 96-well plate and the absorbance was recorded using a ThermoMax microplate reader with a 570/650 nm filter. Cell number was then calculated from a standard curve developed by recording absorbances from known numbers of similar cells prepared in an identical fashion for reading.

Soft Agarose Assay. SeaPlaque agar (1.4%; FMC Bioproducts) in water was mixed with equal amounts of 2× serum-containing medium in a 60-mm dish to form the lower layer. Within 1 h, the upper layer composed of equal

volumes of cell suspension (5×10^4 cells/8 ml) and the lower layer mix was poured at 37°C. NGF was added only to the upper layer. Cultures were fed every 4–5 days with 1 ml of medium with 0.05% agarose, and colonies were counted after 2 weeks. Experiments were performed in triplicate.

Immunohistochemical Procedures. Cells on coverslips were fixed in 4% paraformaldehyde and washed with 1% normal horse serum/0.5% Triton X-100 in PBS for 15 min. The following antibodies were applied: GFAP (Boehringer Mannheim), murine IgG1, 1:500 for 1 h; SMI312 (Sternberger Monoclonals) which is directed against the heavy and medium subunits of neurofilament, murine IgG1, 1:1000 for 1 h; and anti-trkA (TA-1), murine IgG3, 1:250 overnight. Control isotype-matched mabs diluted in PBS at a concentration equal to the primary antibody were always assessed concurrently as negative controls. After incubation in primary antibody, coverslips were rinsed three times in either 1% normal horse serum or 0.5% Triton X-100 buffer. Biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Inc.) was applied for 30 min at room temperature and rinsed for 15 min followed by a 30-min incubation in avidin-conjugated horseradish peroxidase (avidin-biotin complex method; Vector Laboratories, Inc.) and developed in DAB substrate (0.7 mg/ml DAB 2.0 mg/ml urea-H_2O_2, Sigma FAST tablets; Sigma) for 5 min. Coverslips were then air dried and mounted (Permount; Fisher Chemicals). Samples were viewed with a Zeiss Axioskop microscope. Images from color-positive films were digitized with a Nikon Coolscan. Brightness and color were adjusted with Adobe Photoshop, and the color montage was assembled with CorelDraw. The figures were printed using a Kodak Colorease PS printer on Kodak Extatherm XLS paper.

Intracranial Inoculations. In accordance with the University of Massachusetts Medical Center's guidelines concerning usage of small animals, male Wistar-Kyoto rats (Taconic Farms, MA) weighing 200–250 g were anesthetized with 70 mg/kg pentobarbital i.p. and placed in a stereotactic head holder (David Kopf Instruments). A midline incision was made to expose the coronal, sagittal, and lamdoidal sutures and a burr hole was made 3 mm lateral and posterior to the bregma. Tumor cells (5×10^6) in 40 μl of MEM without serum were loaded into a Hamilton syringe which was advanced through the drill hole to a depth of 5 mm. The suspension was then injected over 2 min, after which the syringe was slowly withdrawn and the skin incision was closed with Michel clips. Rats were then assessed daily for signs of illness (lethargy, subconjunctival hemorrhages, weight loss, and gait impairment). For pathological studies, preselected animals were perfused with saline and 4% paraformaldehyde via a transcardiac approach, after which the brains were removed and placed in 0.2 M sodium phosphate buffer containing 30% sucrose. Forty-μm sections were cut on a frozen microtome. Tumor extent was initially assessed by staining sections cut through the needle tract with 1% cresyl violet acetate solution (pH 3.8) for 30 to 60 s. Histochemical studies were then performed in an identical manner to the *in vitro* samples (see above). For nestin staining, a murine IgG1 mab (PharMingen) was used. Images were processed and prepared as described above.

Assessment of Proliferation and Apoptosis. One μg/ml murine anti-proliferating cell nuclear antigen mab (Oncogene Science) was applied overnight at 4°C to sections permeabilized with 0.1% Triton X-100, after which slides were washed three times in PBS and slides were developed with a Vector avidin-biotin kit. To assess the extent of apoptosis, an ApoTag detection kit (Oncor) was utilized. Forty-μm sections were hydrated in PBS and treated with 20 μg/ml proteinase K for 15 min at room temperature. After three washes in distilled H_2O, sections were equilibrated in PBS for 10 min and then endogenous peroxidases were quenched with 1% H_2O_2 in methanol. After two PBS washes, sections were incubated with equilibration buffer for 1 min followed by a 30-min incubation with terminal deoxynucleotidyl transferase at 37°C, and the reaction was terminated with stop solution for 10 min. After three washes in PBS, sections were incubated with anti-digoxygenin peroxidase for 30 min, washed three times in PBS, and developed with the peroxidase reaction using a Sigma DAB substrate kit.

RESULTS

C6 glioma cells, like human glioma lines, do not express trkA (12–14). We transfected C6 cells with expression vectors encoding either a full-length rat trk receptor ($C6^{trkA}$) or a truncated trk receptor which expresses the extracellular and transmembrane domains but not

the intracellular signaling component ($C6^{trkAΔ}$). Receptor expression was documented on $C6^{trkA}$ and $C6^{trkAΔ}$ cells using RT-PCR, Western blotting, and flow cytometry (Fig. 1). The experimental molecular weight of the trkAΔ product is 95,000, in good agreement with the prediction (96,000) from the cDNA sequence. NGF treatment of $C6^{trkA}$ but not $C6^{trkAΔ}$ resulted in tyrosine phosphorylation of the trkA receptor (data not shown). Hence, the trkA receptor expressed in $C6^{trkA}$ cells is functional, whereas that in $C6^{trkAΔ}$ cells is not. The three C6 lines grew equally well in both serum-supplemented and serum-free defined media. Addition of NGF to the medium did not affect proliferation of C6 or $C6^{trkAΔ}$ cells; however, even low doses (100 pg/ml) of NGF increased the growth of $C6^{trkA}$ cells (Fig. 2A), in agreement with the findings of Colangelo *et al.* (13). Furthermore, $C6^{trkA}$ colony formation in soft agarose increased in the presence of NGF ($P < 0.004$; Fig. 2B). Long-term treatment (3 weeks) of $C6^{trkA}$ cells with NGF did not result in dependence on NGF for survival. Additionally, as judged by immunohistochemistry, GFAP, a marker of differentiated astrocytes, was not increased in $C6^{trkA}$ cells, compared to either C6 or $C6^{trkAΔ}$, when cells were incubated in the presence of NGF for 1 week. These cell culture experiments suggested that trkA expression would increase the tumorigenicity of C6 cells.

Since *in vitro* results do not necessarily predict *in vivo* behavior (21), we implanted 5×10^6 C6, $C6^{trkA}$, and $C6^{trkAΔ}$ cells into rat striata. In this bioassay, rats harboring $C6^{trkA}$ implants survived significantly longer than rats inoculated with either of the other two cell lines (Fig. 3). No rat harboring C6 or $C6^{trkAΔ}$ cells survived for 15

A. RT - PCR

- 75 bp

B. Western Blot
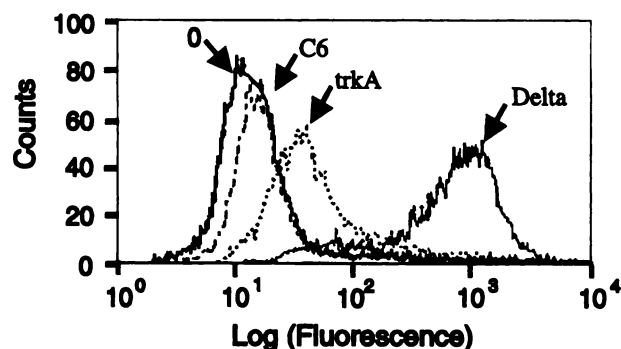

- 140 kDa
- 95 kDa

C. Flow Cytometry

Log (Fluorescence)
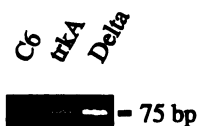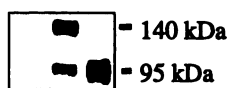
Fig. 1. Expression of trkA by C6 cell lines. *A,* RT-PCR detects 75-bp band characteristic of trkA for $C6^{trkA}$ and $C6^{trkAΔ}$ but not for C6. *B,* TrkA detected by Western blotting. TrkA is not detected for C6 cells, but a M_r 140,000 band and a minor M_r 95,000 band are detected for $C6^{trkA}$ and a M_r 95,000 band is detected for $C6^{trkAΔ}$. The minor M_r 95,000 band for $C6^{trkA}$ is commonly seen for trkA-expressing cells and is probably due to proteolysis. *C,* Flow cytometric analysis of trkA expression on cell lines. *0,* control IgG3 mab (curves for all three lines superimposable). C6, trkA, and Delta represent fluorescent cytograms for anti-trkA mab binding to C6, $C6^{trkA}$, and $C6^{trkAΔ}$ cells, respectively. C6 cells do not specifically bind anti-trkA mab, but both trkA lines do.
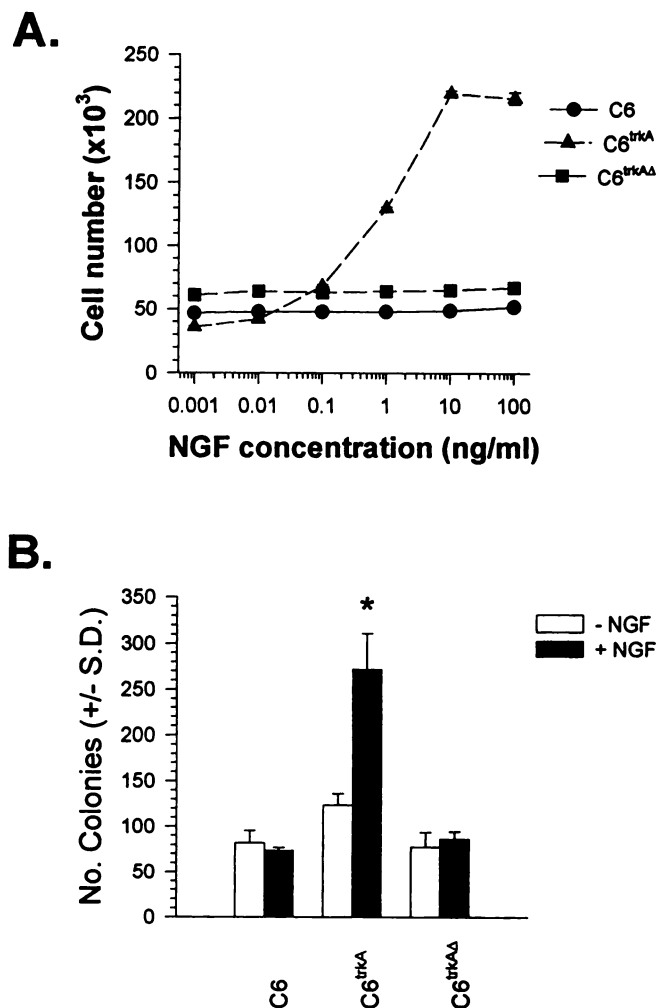
A.

B.

Fig. 2. C6^{trkA} cells are NGF responsive in both MTT and soft agarose assays. *A*, In the MTT assay, 100 pg/ml NGF results in a doubling of C6^{trkA} cells, whereas both C6 and C6^{trkAA} are unresponsive to NGF concentrations a 1000-fold higher. The results represent the result of four experiments; since SEMs are typically <1%, *bars* are undetectable. *B*, In the soft agarose assay, the addition of 100 ng/ml NGF to the media significantly increases colony formation of C6^{trkA} cells (*P* < 0.004, *t* test), but it has no effect on the other two lines.

days postimplantation whereas 54% of the C6^{trkA}-bearing rats survived for longer than 42 days (*P* < 0.001, log rank test). Since rats inoculated with as few as 5 × 10^4 C6 cells expire within 3 weeks, this prolonged survival after a substantial inoculation of C6^{trkA} cells suggests a profound effect of trkA on *in vivo* tumor growth.

Postmortem examination of two long-term surviving rats bearing C6^{trkA} cells revealed evidence of hydrocephalus but no obvious tumor, raising the possibility that C6^{trkA} cells either did not survive implantation or regressed after implantation. To identify mechanisms for increased survival of C6^{trkA}-bearing rats, histological examination of tumor-bearing rat brains was performed. Rats were sacrificed 11 days postinoculation, and brains were prepared for histological analysis. All rats inoculated with C6 or C6^{trkAA} cells had macroscopically large invasive tumors. C6^{trkA} tumors were noted in all animals examined, but these tumors were smaller than those produced by C6 or C6^{trkAA} cells. Hence, expression of trkA appears to influence tumor growth rather than implantation.

Detailed histological examination of tumor-containing brains were carried out to determine the effects of trkA on tumor growth. Staining with anti-trkA (Fig. 4, *A–C*) confirmed that C6 tumors lack trkA, and C6^{trkA} and C6^{trkAA} cells continue to express receptor postimplanta-

tion. C6^{trkA} tumors were distinguished from both C6 and C6^{trkAA} tumors by the lack of infiltration into normal tissue. In brain sections stained with H&E, numerous infiltrating C6 and C6^{trkAA} tumor cells were detected in brain tissue (Fig. 4, *D–F*). In contrast, the C6^{trkA} tumors had a well-defined margin with very few cells in the surrounding tissue. A similar pattern was observed when sections were immunostained for nestin, a primitive neuroepithelial cell marker which is strongly positive in C6 cells but is expressed at very low levels in adult brain (Ref. 22; Fig. 4, *G–I*). Again, we detected many nests of infiltrating tumor cells for C6 and C6^{trkAA} but not for C6^{trkA}.

The decreased invasiveness of C6^{trkA} would explain the longer survival of rats bearing these cells but would not explain the apparent regression of the primary tumors in long-term survivors. For neuroblastomas, trkA expression may lead to regressions by inducing tumor differentiation (23). This mechanism is not applicable to C6^{trkA} cells since GFAP staining of these tumors was negative (Fig. 4, *J–L*). However, while examining tumor cell differentiation, we detected GFAP-positive cells that rimmed C6^{trkA} tumors, demarcating tumor from surrounding brain, and having the appearance of reactive astrocytes. Such a pattern was not seen with the other two lines in which the GFAP staining in the surrounding parenchyma appeared to be more diffuse and disorganized.

Another mechanism by which trkA might effect tumor growth is by influencing rates of tumor cell proliferation and apoptosis. No significant differences in the number of positively stained cells were noted using anti-proliferating cell nuclear antigen as a marker of proliferation (data not shown). In contrast, C6^{trkA} tumors contained a greater number of apoptotic cells than either C6 or C6^{trkAA} (Fig. 4, *M–O*). Statistical analysis of 48 HPFs per tumor type revealed a significant difference in the number of apoptotic cells visualized by terminal deoxynucleotidyl transferase-mediated nick end labeling staining between cell lines. Thus, although the mean number of cells per HPF was 0.3 ± 0.1 and 0.5 ± 0.1 nuclei/HPF for C6 and C6^{trkAA} tumors, respectively, this value was more than 4-fold greater (2.2 ± 0.3, *P* < 3 × 10^{-10}, one-way ANOVA on ranks) for C6^{trkA}. Enhanced apoptosis might lead to slower growth or even regression of C6^{trkA} tumors.
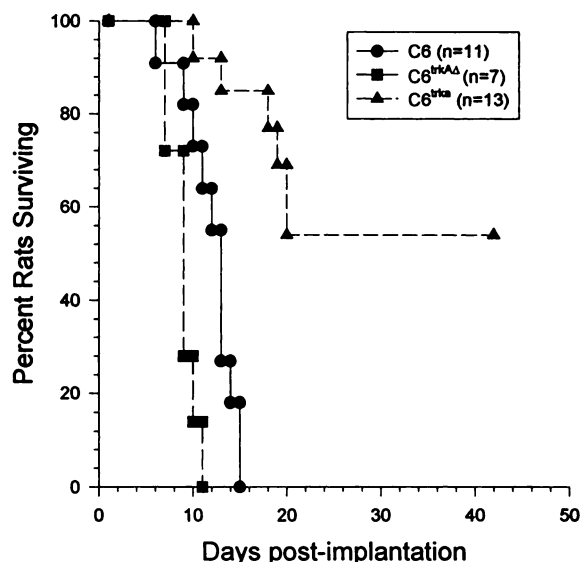

Fig. 3. Survival of rats inoculated with C6 cell lines. After intrastriatal injections of 5 × 10^6 glioma cells, all rats harboring C6 or C6^{trkAA} cells were dead by day 15 (median survival of 12 and 9 days, respectively), whereas 54% of the rats receiving C6^{trkA} cells survived for longer than 42 days (*P* < 0.001, log rank test, C6^{trkA} *versus* both lines). This graph represents the composite of inoculations performed on four separate occasions.
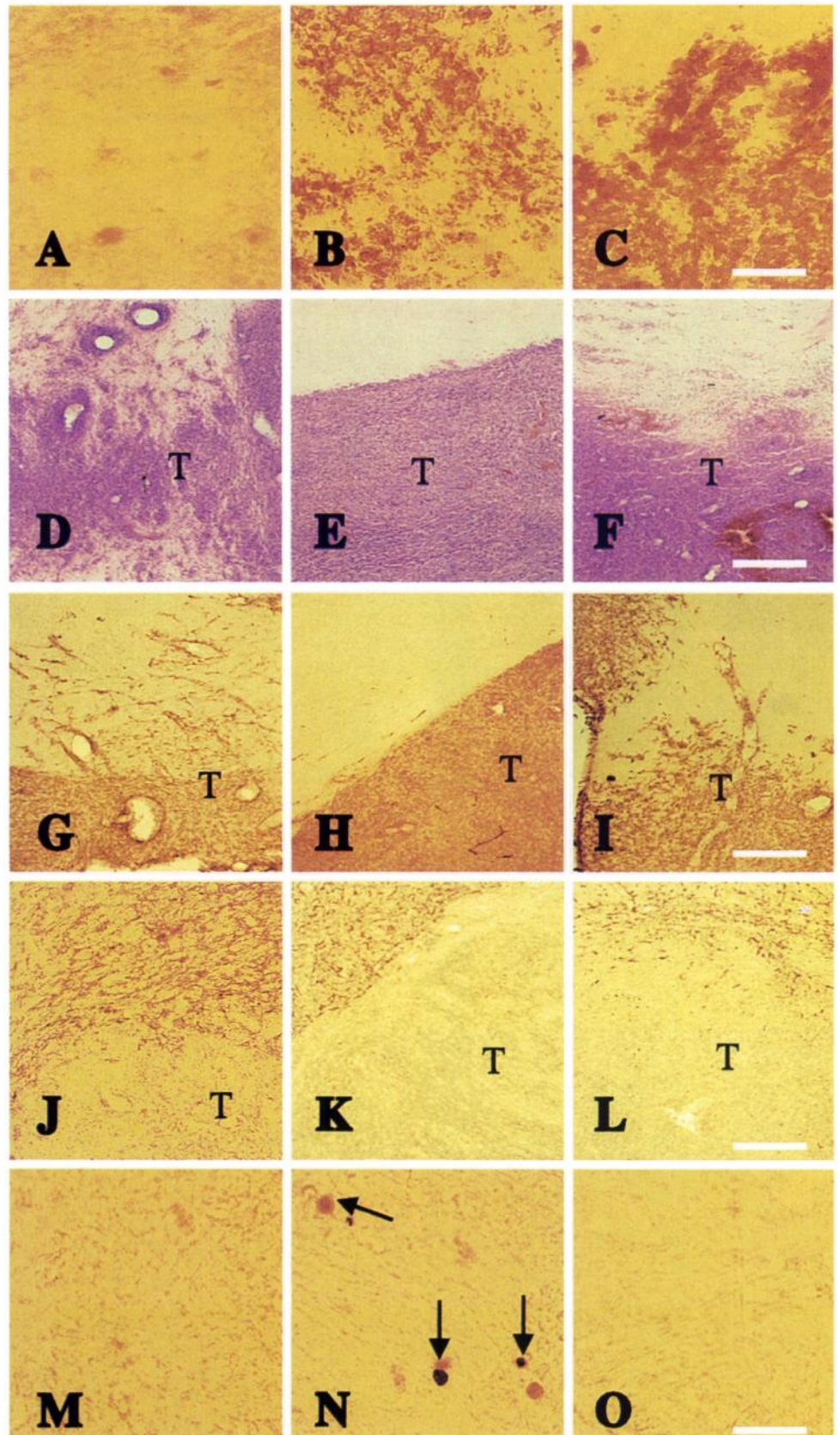
Fig. 4. Histological analyses of brain tumor sections demonstrating trkA expression, decreased invasiveness, and increased apoptosis for C6^{trkA} tumors. Sections of brain tumors from the C6 (A, D, G, J, and M), C6^{trkA} (B, E, H, K, and N), and C6$^{trkA\Delta}$ (C, F, I, L, and O) were stained with anti-trkA mab TA-1 (A–C), H&E (D–F), anti-nestin mab Rat 401 (PharMingen; G–I), anti-GFAP polyclonal antibody (Boehringer Mannheim; J–L), and terminal deoxynucleotidyl transferase-mediated nick end labeling staining for apoptotic damage to DNA (M–O). T, tumor. A–C: bar, 13 μm; D–L: bar, 52 μm; M–O: bar, 9 μm. Arrows in N connote apoptotic cells.

DISCUSSION

The basic finding of this study was that trkA expression modulates the *in vivo* aggressiveness of a highly malignant glioma cell line. Since trkA, but not trkAΔ, enhances survival, the protective effect of

trkA results from the signal-transducing kinase domain. This is not a general effect induced by any receptor kinase because C6 cell lines expressing epidermal growth factor receptor or platelet-derived growth factor receptor are very invasive and result in short survivals

of rats postimplantation (data not shown). Furthermore, the observation that C6^{trkAA} cells produce aggressive tumors which result in short survivals suggest that this effect does not result from an anti-trkA immune response. In contrast to earlier studies with neuroblastoma cells, we find that trkA exerts these effects without inducing differentiation of glioma cells (24).

Glioma invasiveness is an important determinant of clinical outcome; previous studies have indicated that the ability to invade either normal tissue or artificial barriers is correlated with clinically malignant behavior (25). Decreased invasiveness might reflect changes in motility or expression of matrix proteases, both of which are under study in our laboratories. Enhanced apoptosis might also be another mechanism for why trkA-expressing cells are less aggressive; it might reflect an inability to receive sufficient nutrients due to lack of infiltrative capacity or a dependence of the tumor cells on NGF for survival. Although we have not detected increased apoptosis or a dependence on NGF in vitro, it is possible that the C6^{trkA} cells in the brain environment have different requirements for survival.

Could the induction of trkA represent a viable therapeutic strategy for treating malignant gliomas? Motile invading glioma cells represent an important reason why malignant gliomas recur despite visually total resections and are a major reason underlying their grim prognosis (26). Although much research has been directed toward the mechanisms that underlie glioma malignancy, there have been few studies asking why certain glioma types are benign. In the juvenile pilocytic astrocytoma of childhood, for example, neoplastic astrocytes remain localized and confined, making them amenable to resection and long-term survival (1, 2, 4). Although the transformation of a malignant glioma into a benign one might seem less attractive than total tumor eradication, inhibition of glioma cell infiltration, perhaps through induction of trk expression, might greatly improve patient survival.

ACKNOWLEDGMENTS

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