Activation of Neurotrophin-3 Receptor TrkC Induces Apoptosis in Medulloblastomas


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ABSTRACT

Elevated expression of the neurotrophin-3 (NT-3) receptor TrkC by childhood medulloblastomas is associated with favorable clinical outcome. Here, we provide evidence that TrkC is more than simply a passive marker of prognosis. We demonstrate that: (a) medulloblastomas undergo apoptosis in vitro when grown in the presence of NT-3; (b) overexpression of TrkC inhibits the growth of intracerebral xenografts of a medulloblastoma cell line in nude mice; and (c) trkC expression by individual tumor cells is highly correlated with apoptosis within primary medulloblastoma biopsy specimens. TrkC-mediated NT-3 signaling promotes apoptosis by activating multiple parallel signaling pathways and by inducing immediately early gene expression of both c-jun and c-fos. Considered collectively, these results support the conclusion that the biological actions of TrkC activation affect medulloblastoma outcome by inhibiting tumor growth through the promotion of apoptosis.

INTRODUCTION

Medulloblastoma, a malignant tumor of the cerebellum, accounts for ~20% of primary brain tumors in children (1). A high percentage of medulloblastomas express neuron progenitor markers and cerebellar granule cell-specific transcription factors, including PAX6, EN2, and Zic, suggesting that they arise by oncogenic transformation of granule cell precursors (2–8). Medulloblastomas also express neurotrophins that regulate granule cell development, including BDNF and its specific receptor tyrosine kinase TrkB, as well as NT-3 and its preferred receptor TrkC (9–12). During normal development, BDNF promotes survival and differentiation of early postmitotic granule cells (13–16), whereas mature granule cells express increased amounts of TrkC through which NT-3 promotes axonal maturation (16). The role of these developmentally expressed molecules in medulloblastoma oncogenesis is not known.

Patients with medulloblastoma have a bimodal outcome. Despite extensive treatment with surgical excision, external beam irradiation including the entire craniospinal axis, and multiple drug chemotherapy, their prognosis is not uniformly favorable (17–19). Sixty to 80% of children survive 5 years after diagnosis and relapse infrequently thereafter (20, 21), but the remainder relapse and usually die despite receiving identical therapy. We have shown that expression of the NT-3 receptor TrkC in medulloblastomas correlates with favorable clinical outcome (12), indicating that they are possibly derived from more fully differentiated granule cells. Here, we investigate the biological actions of TrkC that may account for the improved prognosis of patients with medulloblastomas that express high levels of the receptor.

MATERIALS AND METHODS

Patient Data and Human Tumor Bank. All patients treated for medulloblastoma between June 1993 and September 1997 at Boston Children’s Hospital and the Dana-Farber Cancer Institute were included in the study (n = 28). In addition, 10 samples were obtained from patients at New York University Medical School, and 4 were from the New England Medical Center. The samples were immediately snap-frozen in liquid nitrogen and stored at −80°C and also transported in medium and processed for tissue culture. In all cases, the diagnosis of medulloblastoma was confirmed by pathological analysis of biopsy samples. At the time of diagnosis, the patients ranged in age from 7 to 324 months (mean = 107 months). There were 14 females and 28 males. All patients were treated with craniospinal irradiation to 2400–3600 cGy with a tumor dose of 5300–7200 cGy. All but three patients were treated with chemotherapy consisting of cisplatin or carboplatin and combinations of vincristine, etoposide, cyclophosphamide, or lomustine. Two patients received high-dose chemotherapy, one as primary therapy and the other at relapse, including methotrexate and thiotepa, followed by autologous bone marrow transplantation. The median survival for the entire group was 67 months (follow-up range: 3–102 months).

Cell Culture and Transfections. To test for neurotrophin responsiveness, freshly obtained tumor biopsy samples were minced, triturated, and grown as suspensions in serum-free DMEM (Cellgro; Mediatech, Herndon, VA) containing high glucose (6 g/liter) and either NGF, BDNF, or NT-3 (50 ng/ml) from stock solutions containing 0.1 mg/ml BSA; neurotrophins courtesy of Andrew Welcher, Amgen, Thousand Oaks, CA) or mock-stimulated control (0.1 mg/ml BSA) at 37°C in 5% CO2. Suspension cultures were stimulated for up to 48 h, fixed in 4% paraformaldehyde or formaldehyde in PBS, and dried onto glass microscope slides. Cells were then stained with the fluorescent nuclear dye bis-benzimide (Hoechst 33342 (10 µM in PBS); Sigma Chemical Co., St. Louis, MO), and the proportion of apoptotic cells was counted in 5–10 high-powered fields for each of four replicated coverslips per condition through a fluorescence microscope (Leitz; ×100 objective) with the observer blinded to the experimental conditions. Other tumor specimens were prepared and cultivated as monolayers on PLL-coated glass coverslips (Bellico Glass, Vineland, NJ) in MEM (Cellgro) containing 12% heat-inactivated FCS (Sigma), high glucose (6 g/liter), 25 mM potassium chloride, sodium pyruvate, and nonessential amino acid supplements (Life Technologies, Inc., Gaithersburg, MD). For monolayer cultures, medium was changed after 24 h in vitro to serum-free MEM containing individual neurotrophins (see above). The coverslips were fixed for an additional 48 h before fixation in 4% paraformaldehyde in PBS, stained with bis-benzimide, and viewed with a fluorescence microscope using the methods noted above.

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The abbreviations used are: BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NGF, nerve growth factor; PLL, poly-L-lysine; CHX, cycloheximide; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal protein kinase; SAPK, stress-activated protein kinase; PI3-K, phosphatidylinositol-3′-kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NSE, neuron-specific enolase; IEG, immediate-early gene.
The medulloblastoma cell line Daoy was obtained from the American Type Culture Collection and maintained in DMEM with high glucose (6 g/liter), 2 mm L-glutamine, and 10% (vol/vol) FCS at 37°C in 5% CO2. Transfected Daoy subclones with increased TrkC expression (Daoy-trkC) were maintained in selective media containing Geneticin (G418, 200 μg/ml; Gibco BRL Life Sciences, Inc.) after stable lipid-mediated transfection with an expression plasmid encoding full-length rat gp145ERK (TrkC; courtesy of Luis Parada, University of Texas; Ref. 22) using Lipofectin, according to vendor recommendations (Life Technologies, Inc.). To test for neurotrophin responsiveness, we grew cell lines on PLL-coated glass cover slips that were placed in serum-free DMEM for up to 96 h with individual neurotrophins (50 ng/ml NGF, BDNF, or NT-3) or vehicle control (0.1 mg/ml BSA). For signal transduction and Western blot experiments, transient stimulations were performed for 30–60 min and then replaced with serum-free media and incubated until fixation and staining, as described above.

Growth curves of Daoy and Daoy-trkC cell lines were determined using trypsin blue exclusion (23). Proliferation of cell lines was measured by determining incorporation of 6-[3H]thymidine (>10 Ci/mmol; New England Nuclear, Boston, MA; Ref. 24). Briefly, parallel Daoy-trkC cultures were prepared, stimulated, and incubated for the last 6 h with [3H]thymidine-containing medium (60 μCi/ml). Cells were then harvested using 0.1% Triton X-100, and trichloroacetic acid-precipitable 3H counts were quantitated with a scintillation counter.

Pharmacological inhibitors of signal transduction K-252A (Kamiya Biochemical, Seattle, WA; wortmannin, PD98059, and SB230850 (Calbiochem, San Diego, CA), or CHX (Sigma) were also added to select cultures in serum-free DMEM or MEM at indicated concentrations for 30 min prior to and during neurotrophin or mock stimulation.

**RNA Isolation and Northern Analysis.** Total cellular RNA was isolated by cesium chloride gradient ultracentrifugation after tissue homogenization in guanidine isothiocyanate-containing lysis buffer. Northern blotting, including hybridization conditions and RNA quantification, were performed as described previously (12). All signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and corrected for variations in sample loading by comparison to internal 28S rRNA expression. RNA expression indices for cell culture experiments were calculated by normalizing samples to their 28S rRNA content and by comparing expression by steady-state unstimulated cells (25). Trk probes were prepared by random oligonucleotide-primed 32P-labeling of cDNAs encoding the extracellular domains of human TrkB and TrkC (courtesy of David Shelton, Genentech, San Francisco, CA). The 2.6-kb c-jun cDNA probe was similarly prepared from a previously described plasmid (26). All other probes have been described previously (12).

**Western Analysis, Immunoprecipitation, and Immunohistochemistry.** Cellular lysates were prepared from transiently stimulated Daoy-trkC cultures or fresh tissue by lysis in NP40-glycerol lysis buffer and from frozen tissue samples by rapid boiling in SDS-lysis buffer, as described previously (12, 27). For signal transduction and Western blot experiments, transient stimulations were performed for 30–60 min and then replaced with serum-free media and incubated until fixation and staining, as described above.

Separated proteins were then transferred to nylon membranes (Immobilon-P; Millipore, Bedford, MA) for immunoblotting as described previously (29). Specific antibodies included: an antiphosphotyrosine monoclonal (4G10; Upstate Biotechnology Inc., Lake Placid, NY); an anti-p70S6K polyclonal (courtesy of M. Greenberg and J. Blenis; Ref. 30); an anti-Erk1/2 (p44ERK/p42ERK) polyclonal generated against a COOH terminus peptide (cMAPK; courtesy of J. Blenis; Ref. 31); an anti-Erk2 monoclonal (1B3B; courtesy of M. Greenberg and M. Weber; Ref. 32); a phospho-Tyr-204-specific antibody for Erk1/2, a phospho-Thr-183 and phospho-Tyr-185-specific antibody for JNK/SAPK, and a phospho-Tyr-182-specific antibody for p38MAPK (homologous to yeast Hog1 kinase; New England Biolabs, Beverly, MA); and a phospho-Ser-73-specific polyclonal for c-Jun (courtesy of M. Greenberg). Membranes were blocked using either 4% BSA or 5% BSA, incubated, and washed using standard techniques (29). Secondary antibodies included goat antimumus IgG- and goat antirabbit IgG-conjugated to alkaline phosphatase for chemiluminescent detection with CDP-Star reagents, according to vendor recommendations (New England Biolabs).

Immunoprecipitations of PI3K and ERK1/2 from cellular lysates were performed using antibodies against antiphosphotyrosine (4G10) and the COOH terminus of ERK1/2 (cMAPK), respectively, with protein A-Sepharose beads (4CLB; Pharmacia, Piscataway, NJ) for SDS-PAGE analysis, as described previously (27, 33). Briefly, for in vitro kinase assays of PI3’K and ERK1/2 activities, cellular lysates were prepared at indicated time points after transient stimulation as above and then incubated, as described previously (27, 33), with γ[32P]ATP (3000 Ci/mmol; New England Nuclear, Boston, MA), and the appropriate substrate (phosphotylinositol and phosphatidylerine) or rabbit myelin basic protein (Sigma). For PI3’K assays, radiolabeled phospholipids were extracted, separated with TLC, and quantitated with a PhosphorImager. For ERK assays, radiolabeled myelin basic protein was precipitated with trichloroacetic acid and immobilized on phosphocellulose filters (P81; Whatman, Hillsboro, OR), washed in dilute phosphoric acid, and quantitated with a scintillation counter.

Immunoprecipitations of c-Fos, Daoy-trkC cultures were grown in PLL-coated coverslips and stimulated as above. Following fixation and incubation with an anti-c-Fos polyclonal antibody (courtesy of M. Greenberg), a peroxidase-antiperoxidase system was used according to the manufacturer’s recommendations (Vectorstain; Vector Laboratories, Burlingame, CA).** In Situ Hybridization.** Paraffin-embedded 10-μm-thick tumor sections were hybridized with 32P-labeled antisense and sense human Trk riboprobes (see above) according to a previously described protocol (34). The tissue sections were hybridized at 45°C for 18–20 h, dipped in photographic emulsion (NTB; Kodak, Rochester, NY), exposed at 4°C for 7 days, developed, counterstained with hematoxylin, and viewed with bright- and dark-field microscopy.

**Apoptosis and Proliferation Analysis.** Hematoxylin-stained biopsy sections were also examined to define regions with dense infiltration of tumor cells. Comparable fields in adjacent serial sections were examined for apoptosis and for proliferation. The proportion of proliferating cells was determined in tissue sections stained with a Ki-67 antibody (DAKO, Carpinteria, CA) and visualized using peroxidase-antiperoxidase techniques, per vendor recommendations. For detection of apoptosis, the paraffin sections from the same patients and fixed monolayer cell cultures were assessed for nucleosomal degradation by TUNEL using digoxigenin-labeled nucleotide incorporation, a fluorescein-labeled antidigoxigenin antibody, or peroxidase-antiperoxidase techniques, according to the manufacturer’s recommendations (Aptopt; Oncor, Gaithersburg, MD). The sections were counterstained with propidium iodiode or bisbenzimide. Nuclei were counted through a fluorescence microscope under high power (Leitz, ×100 objective). In a separate set of observations, the proportion of pyknotic, condensed, and fragmented nuclei in propidium iodiode-stained sections were counted (×40 objective). Nuclei were counted in 10 high-powered fields per tumor biopsy sample by blinded observers to determine the fraction of nuclei undergoing apoptosis. For the purposes of statistical analysis, we calculated an apoptosis index by normalizing the proportion of apoptosis for each condition tested to the percentage observed in a parallel mock-stimulated control performed in each experiment.

**Medulloblastoma Cell Line Intracranial Xenografts in Nude Mice.** Equal numbers (1 × 106 cells in 5 μl) of the trkC-overexpressing Daoy cells (Daoy-trkC) and control parental Daoy cells were each injected into opposite cerebral hemispheres of nude mice (nu/nu Balb/C adult males anesthetized with Avertin, 0.02 ml/g body weight) using a Hamilton syringe guided by a stereotaxic apparatus (Stoeling, Wood Dale, IL). Injections were made 3 mm caudal to Bregma, 2.5 mm lateral of midline, and 3 mm below the surface of the brain. The animals were then allowed to recover, and after 7–9 weeks were anesthetized and sacrificed by intracardiac perfusion with PBS followed by 4% paraformaldehyde in PBS. Serial 15-μm sections were cut on a cryostat, mounted on glass slides, stained, and viewed with a bright-field microscope. To measure tumor volumes, the tumors in each section were traced, and cross-sectional area was calculated on a videomicroscopy image analysis system (Image-1; Universal Imaging, West Chester, PA). The volume per section was calculated by multiplying the area by section thickness, and the total tumor volume was calculated by summation of the tumor section volumes in each hemisphere.

**Statistical Analysis.** Kaplan-Meier survival analysis was calculated with SAS (SAS Institute, Inc., Cary, NC) using the log-rank test for comparisons (35). Cell counting data were tested for normality with the Kolmogorov-Smirnov test and evaluated by ANOVA (Stat-View; Abacus Concepts, Inc., Berkeley, CA) using the Bonferroni procedure for multiple post hoc pairwise tests.
by normalizing the 14.0-kb \textit{trkC} transcript signal to a standardized mouse brain RNA control (25). The expression of \textit{trkC} was highly variable in medulloblastoma specimens (expression index range, 0.1–45.7; median = 0.80; mean = 3.9). \textit{trkC} expression in normal human cerebellum was determined to be 2.0 times higher than mouse brain. The cohort of patients was dichotomized into groups expressing high or low levels of \textit{trkC} by dividing at the median index value (overall trends are similar using the mean index). Survival data compiled in Fig. 1 and Table 1 reveal that patients with medulloblastomas expressing high levels of \textit{trkC} had more favorable progression-free survival (Fig. 1B) and overall survival (Fig. 1C; median survival = 92 months) compared to those with low \textit{trkC} expression (median survival = 39 months). Fewer patients with high \textit{trkC}-expressing tumors had evidence of metastatic disease at the time of diagnosis (2 of 20 patients) than those (10 of 22 patients) with low \textit{trkC} expression, although the trend was not statistically significant (Fisher’s exact test, \( P = 0.09 \)).

The expression of other neurotrophins and their receptors was not predictive of outcome (Table 1). Neither patient age nor the extent of surgical resection correlated with \textit{trkC} expression. Moreover, age, degree of surgical resection, sex, and treatment center were not predictive of survival. Considered collectively, these data indicate that \textit{trkC} expression was the only parameter studied that served as a significant predictor of clinical outcome.

**NT-3-stimulated TrkC Activation Induces Cell Death in Primary Medulloblastomas in Vitro.** We initially addressed whether TrkC activation alters medulloblastoma growth by adding NT-3 to primary cultures of medulloblastomas \textit{in vitro}. Medulloblastoma cultures from two patients with high \textit{trkC} expression and another with low \textit{trkC} expression were grown under serum-free conditions in the presence of NT-3, BDNF, or NGF (each at 50 ng/ml) for 18–48 h. Upon microscopic examination, some of the cells in each culture revealed nuclear pyknosis, condensation, or fragmentation indicative of cell death. Cultures from all three medulloblastomas displayed a marked increase of apoptosis when grown in the presence of NT-3 (Fig. 2B) compared to mock-stimulated controls (Fig. 2A), but not in BDNF or NGF (\( P < 0.0001 \), ANOVA; Fig. 2D), indicating that NT-3 binding to its preferred receptor, TrkC, increased cell death. This effect was blocked by CHX in the one tumor tested (Fig. 2C). Thus, medulloblastomas grown in culture respond to TrkC activation by undergoing apoptosis.
TrkC Activation Induces Apoptosis of the Daoy Medulloblastoma Cell Line in Vitro. To test whether we could induce TrkC-mediated apoptosis in tumor cells that normally do not respond to NT-3, we up-regulated the expression of the TrkC receptor in a medulloblastoma cell line that normally has no detectable expression of full-length p145TrkC (TrkC). The human medulloblastoma-derived Daoy cell line normally expresses very low levels of the 5.5-kb trkC alternate splice variant and no detectable 14-kb trkC, p75NTR, trkA, or trkB mRNA (12). We stably transfected Daoy cells with an expression plasmid encoding full-length TrkC and tested subclones (designated Daoy-trkC) for increased TrkC protein expression by immunoprecipitation and for TrkC receptor activity using immunocytochemistry to detect NT-3-induced c-Fos expression (see below; Ref. 36). The levels of trkC mRNA expression of two Daoy-trkC subclones were 4.6 and 6.6 times greater than mouse brain and, therefore, ~2–3 times greater than normal human cerebellum.

When grown in serum-free conditions, the Daoy-trkC subclones overexpressing trkC underwent extensive cell death in the presence of NT-3 (Fig. 3B) compared to mock-stimulated controls (Fig. 3A) but not BDNF or NGF (50 ng/ml each; P < 0.0001; Fig. 3E). Apoptosis was morphologically evident within 24 h of exposure to NT-3 and increased significantly by the 48- and 72-h time points. By 96 h, the remaining adherent monolayer cells in the NT-3 conditions had ~10-fold greater nuclear pyknosis and fragmentation [51.7 ± 0.4% (mean ± SE); Fig. 3F] compared to mock-stimulated control conditions (5.1 ± 0.3%; Fig. 3A), which was statistically significant [apoptosis index = 10.1 ± 0.08 (mean ± SE); P < 0.0001; Fig. 3E].
To demonstrate that NT-3-induced cell death was due to apoptotic mechanisms, we examined Daoy-trkC cultures for nucleosomal degradation characteristic of apoptosis. The proportion of TUNEL-positive nuclei was significantly higher in Daoy-trkC cultures grown with NT-3 for 24 h [0.08 ± 0.03; (mean ± SE); Fig. 3D] compared to control mock-stimulated conditions in which no TUNEL-positive cells were present (P < 0.03; Fig. 3C).

NT-3/TrkC-induced apoptosis was not observed in the presence of FCS in culture media (Fig. 3E), suggesting that serum contains protective factor(s). In Daoy-trkC cells, neither FCS nor NT-3 induced morphological changes suggestive of differentiation. Similarly, Western blot analysis of NT-3-stimulated Daoy-trkC cell lysates did not reveal changes in the expression of markers such as NSE or microtubule-associated protein-2 isoforms (data not shown).

Growth curves of NT-3-stimulated Daoy-trkC cultures (Fig. 3F) revealed decreasing cell numbers (by approximately one log unit) with increasing duration of NT-3 stimulation, in contrast to stable cell numbers in parallel mock-stimulated Daoy-trkC cultures (Fig. 3F). Proliferation, as determined by \(^{3}H\)thymidine incorporation rates, also progressively decreased in NT-3-stimulated Daoy-trkC cultures, compared to parallel mock-stimulated Daoy-trkC cultures (Fig. 3F). The decreased growth and proliferation rates in NT-3-stimulated Daoy-trkC cultures indicate that NT-3 induces apoptosis without increasing proliferation. These results support apoptosis rather than proliferation or differentiation as the primary effect of NT-3/TrkC signaling on Daoy cell growth in vitro.

**TrkC Signal Transduction Activates PI3'K, ERK1/2, p38MAPK, and JNK/SAPK and Expression of the IEGs c-fos and c-jun.** Because NT-3-induced apoptosis might have been due to defective signal transduction by overexpressed TrkC, we sought to confirm the integrity of NT-3/TrkC signaling pathways. The tyrosine kinase function of activated TrkC receptors in Daoy-trkC cells appears necessary for apoptosis induction because there was no increase in cell death over mock-stimulated cultures when the Trk tyrosine kinase inhibitor K252a (200 nM) was added along with NT-3 (P > 0.4 for both subclones; data not shown).

Further studies of NT-3-stimulated TrkC transduction were then undertaken to confirm that downstream signaling cascades were functionally intact. Daoy-trkC cultures were prepared as described above but were only transiently stimulated with NT-3 for 30–60 min; then the NT-3 was replaced with serum-free medium, and cultures were harvested at regular intervals. Using phosphospecific antibodies in Western blots of these Daoy-trkC lysates, we detected activation (phosphorylation) of known signaling intermediates by PI3'K. Normally, PI3'K phosphorylates the serine-threonine protein kinase Akt, and ribosomal S6 subunit kinase, p70S6Rsk. Using p70S6Rsk-specific antibody in Western blots of NT-3-stimulated Daoy-trkC cell lysates, mobility shifts of p70S6Rsk were detectable for 6 h after transient stimulation (Fig. 4A), consistent with phosphorylation by PI3'K.

Western blots of Daoy-trkC lysates also revealed phosphorylation patterns indicating NT-3-induced activation of three parallel MAPK signaling cascades: ERK (p44/p42), JNK/SAPK, and p38MAPK. An antibody specific for phospho-Tyr-204 of ERK1/2 demonstrated prolonged activation of ERK1/2 (Fig. 4B), whereas no changes in the overall quantity of ERK was detected by a phosphorylation-independent monoclonal antibody (1B3B9; Fig. 4C). As shown by a phospho-Thr-183/185-specific JNK/SAPK antibody, JNK/SAPK is phosphorylated for several h when transiently activated by NT-3/TrkC (Fig. 5D), p38MAPK is more transiently activated by NT-3/TrkC, as indicated by phospho-Tyr-182-specific antibody (lasting ~1 h; Fig. 5A). The PI3'K and ERK1/2 Western blot results were verified directly using in vitro kinase assays. After 1 h of NT-3 stimulation, PI3'K and ERK1/2 activity increased 2.31- and 2.66-fold, respectively, in immunoprecipitated Daoy-trkC lysates compared to mock-stimulated controls.

For a variety of neural cells, activation of PI3'K, ERK1/2, JNK/SAPK, or p38MAPK have been linked to cell death or survival (37–43). Blocking either PI3'K signaling with wortmannin (100–200 nM) or ERK1/2 signaling by inhibiting upstream MEK1/2 with PD98059 (10–50 \( \mu \text{M} \)) completely prevented apoptosis in NT-3-stimulated Daoy-trkC cells (Fig. 6, A and B), indicating that p38MAPK activity is required for NT-3-induced apoptosis (Fig. 6, A and B). Both p38MAPK and JNK/SAPK pathways have been shown to converge on the IEGs c-jun and c-fos (44). We found by probing Northern blots that TrkC activation induced sustained up-regulation of c-jun transcription (Fig. 5D). Moreover, mobility shifts of c-Jun (M, 44,000–45,000) bands were evident on Western blots probed with a phospho-Ser-73-specific c-Jun antibody (Fig. 5C), indicating activation by posttranslational phosphorylation of serine residue 73 by JNK/SAPK (45, 46). Finally, NT-3-induced nuclear expression of c-Fos in Daoy-trkC cells was demonstrated by immunohistochemical methods using c-Fos-specific antibodies (Fig. 5, E and F).

**CHX Blocks NT-3/TrkC-induced Apoptosis in Medulloblastoma.** To test for the possible requirement for protein translation in NT-3/TrkC-stimulated apoptosis, Daoy-trkC cells were incubated with CHX at concentrations (1–5 \( \mu \text{g/ml} \)) sufficient to reduce protein synthesis approximately 50%, as determined by \(^{35}\text{S}\)methionine/cysteine incorporation (data not shown). In the presence of CHX (1 \( \mu \text{g/ml} \)), NT-3-stimulation did not increase apoptosis (Fig. 6, C and F). Cytotoxic effects of CHX in dose ranges used were not evident during these experiments, and Western blots of Daoy-trkC lysates did not reveal CHX-induced changes in signaling intermediates (data not shown).

![Fig. 4](image-url)
TrkC Expression Inhibits the Growth of Daoy Medulloblastoma Cell Line in Vivo. We tested the biological significance of NT-3-TrkC-induced apoptosis in vivo by examining the growth of intracerebral xenografts of Daoy and Daoy-trkC cells in nude mice. We first tested whether there are inherent differences of proliferation in vitro, the growth curve of parental Daoy cells was indistinguishable from Daoy-trkC cells.

Equal numbers of Daoy and Daoy-trkC cells were injected into opposite hemispheres of the brains of anesthetized nude mice and the animals were allowed to recover. After 7–9 weeks, marked differences in the size of the xenografts were evident. In 8 of 10 animals, the intracerebral control Daoy tumors were substantially larger compared to Daoy-trkC tumors from matched opposite cerebral hemispheres (P < 0.03, Wilcoxon rank sum test; Fig. 7, B and C). These differences were most notable after 9 weeks of xenograft growth; the parent Daoy tumors were 7–200-fold larger than the Daoy-trkC tumors. Histologically, both Daoy and Daoy-trkC tumors were highly cellular and without morphological evidence of differentiation. These sections were also examined for apoptosis by TUNEL staining, which revealed dramatically greater proportions of apoptotic nuclei in the Daoy-trkC tumors compared to parental Daoy tumors (Fig. 7, D and E). These results demonstrate that, in our in vivo nude mouse model, trkC overexpression inhibited tumor growth in association with increased apoptosis.

trkC Expression by Medulloblastoma Tumor Cells Is Associated With Apoptosis. We then addressed whether TrkC-induced apoptosis might have growth-inhibiting effects on human medulloblastomas in

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**Fig. 6.** NT-3-induced apoptosis in Daoy-trkC cells is blocked by SB230850 or CHX. Daoy-trkC cells were grown, stimulated, fixed, stained with bis-benzimidazole, and examined using fluorescence microscopy. Scale bars, 10 μm. A and B, mock-stimulated Daoy-trkC control cultures (A) show minimal nuclear changes compared to NT-3-stimulation (50 ng/ml) of Daoy-trkC cells (B), which caused a marked increase of pyknotic nuclei after 72 h (nuclei indicated by arrows). C, NT-3-induced apoptosis was blocked by CHX (1 μg/ml) in Daoy-trkC cultures. D, incubation of NT-3 (50 ng/ml) stimulated Daoy-trkC cultures with p38MAPK inhibitor SB230850 (25 μM) significantly reduced apoptosis compared to NT-3 alone (E). Addition of PD98059 (10 μM), which blocks MEK1/2 upstream of ERK1/2, to NT-3 (50 ng/ml) stimulated Daoy-trkC did not block NT-3-induced cell death compared to NT-3 alone (F). Bar graph summarizing four experiments with Daoy-trkC cells showing the effects of various signal transduction inhibitors [Wort, wortmannin (100 μM); PD, PD98059 (10 μM); SB, SB230850 (25 μM)] and CHX. An apoptosis index was calculated by normalizing data (from five high-powered fields from each of four replicated coverslips per experimental condition) to the proportion of apoptotic nuclei in mock-stimulated controls (3.4 ± 0.3%). The apoptosis index from NT-3-stimulated Daoy-trkC cells (10.71 ± 0.45) was significantly higher than that of mock-stimulated controls (P < 0.0001; ANOVA), and the effect was completely blocked by the presence of CHX (apoptosis index = 1.08 ± 0.08) or SB230850 (apoptosis index = 1.53 ± 0.20; P < 0.0001 for each) but not by wortmannin (apoptosis index = 6.88 ± 1.57) or PD98059 (apoptosis index = 6.53 ± 0.83). Columns, means; bars, SE.

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**Fig. 5.** NT-3/Tak signaling in Daoy-trkC activates p38MAPK and JNK/SAPK, c-Jun phosphorylation, c-Jun transcription, and c-Fos nuclear translocation. Western blots were prepared from Daoy-trkC lysates harvested after transient NT-3 (Lanes +) or mock (Lanes –) stimulation at times indicated, separated by SDS-PAGE, transferred to nylon membranes, blocked with BSA or Blotto, and incubated with primary antibodies including phosphospecific rabbit anti-p38MAPK, anti-JNK/SAPK, or anti-c-Jun primary antibodies. Open arrowheads, phosphorylated isoforms; closed arrowheads, hypophosphorylated forms. A, p38MAPK is transiently activated in NT-3-stimulated lysates by phosphorylation at Tyr-182, as revealed by phosphospecific antibody. B, NT-3 induces sustained phosphorylation of JNK/SAPK species (p46 and p54) at Thr-183 and Tyr-185, as detected by phosphospecific antibody. C, the substrate of JNK/SAPK, c-Jun, is also phosphorylated at Ser-73 in response to NT-3 stimulation, as revealed by phosphospecific antibody staining of mobility shifted upper bands (open arrowheads). Lesser degree of nonspecific phosphorylation is also detected in mock-stimulated lysates. D, trkC activation induces up-regulation of c-Jun mRNA expression. Northern blots of total cellular RNA prepared from transiently stimulated (60 min) Daoy-trkC cultures were hybridized with 32P-labeled c-Jun probe revealing splice variants (arrows). An index of expression was calculated by correcting for 28S mRNA content and normalizing to baseline unstimulated levels (harvested at time 0). These results were replicated in four separate experiments. E and F, Daoy-trkC cultures grown on PLL-coated coverslips, stimulated, and processed for immunohistochemical staining with c-Fos-specific antibody reveal NT-3-induced expression of c-Fos (F) but no induction in mock-stimulated Daoy-trkC controls (E). Scale bar, 10 μm. 

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Fig. 7. Daoy-\(trkC\) xenografts demonstrate diminished growth and increased apoptosis in vivo compared to the parental Daoy cell line. A, growth curves reveal similar growth kinetics of parental Daoy (C) and Daoy-\(trkC\) (X) cell lines cultivated in vitro. B, scatter plot summarizing data from a total of 10 animals, sacrificed at 7 and 9 weeks postinjection, shows the range of differential xenograft growth. Data points, ratios of the volume of the parental Daoy xenograft compared to the volume of the contralateral Daoy-\(trkC\) xenograft from a single animal (i.e., a ratio of 1.0 represents equal-sized xenografts). Equal numbers of Daoy-\(trkC\) and control parental Daoy cells were each injected into opposite cerebral hemispheres of anesthetized nude mice using a Hamilton syringe guided by a stereotaxic apparatus. The animals were then allowed to recover, and after 7–9 weeks were anesthetized and sacrificed by intracardiac perfusion with PBS followed by 4% paraformaldehyde in PBS. Serial 15-\(\mu\)m cryostat sections were mounted, stained, and viewed with a bright-field microscope. The volume of tumors in each section were calculated on a videomicroscopy image analysis system, and the total tumor volume calculated by summation of the tumor section volumes in each hemisphere. C, hematoxylin-stained section reveal significantly greater growth of a parental Daoy xenograft in the left hemisphere (indicated by closed arrowheads) compared to a Daoy-\(trkC\) xenograft in the contralateral (right) hemisphere (indicated by open arrowheads) of a homozygous nude mouse. (X5.25 objective; scale bar, 500 \(\mu\)m). D and E, TUNEL staining of Daoy (D) and Daoy-\(trkC\) (E) xenografts (visible at the bottom portion of each panel) and less densely cellular cerebral parenchyma (at the top of each panel; +) reveals significantly more apoptotic nuclei in the Daoy-\(trkC\) xenograft (arrowheads in E) compared to the parental cell line Daoy xenograft (D), which does not express TrkC. The TUNEL staining was visualized using peroxidase-antiperoxidase methods, and the sections were counterstained with methyl green. X20 objective; scale bar, 50 \(\mu\)m.

Fig. 8. Apoptosis correlates with \(trkC\) expression in primary human medulloblastomas. Primary human tumor specimens were prepared as described in “Materials and Methods” and processed for \(in situ\) hybridization (A–C) and TUNEL staining (D and E). A low \(trkC\)-expressing tumor is shown in A and D, in comparison to a high \(trkC\)-expressing tumor in B and E. Cells from adjacent normal cerebellum are shown in C. A–C, \(in situ\) hybridization of medulloblastoma biopsy samples with a riboprobe (RNA) complementary to \(trkC\) sequences. A section from a primary tumor expressing low levels of \(trkC\) (A), as determined by Northern analysis, had a significantly lower density of silver grains in the emulsion above above the neoplastic cells compared to a section from a tumor expressing high levels of \(trkC\) (B). Expression of \(trkC\) in cerebellar parenchyma (C) is shown within the same tissue section as the high \(trkC\)-expressing tumor. X100 objective; scale bars, 10 \(\mu\)m. D and E, TUNEL staining of cells from low- (D) and high (E) \(trkC\)-expressing tumors reveals increased apoptotic nuclei in the tumor expressing high levels of \(trkC\) (arrows in E). X40 objective, scale bars, 25 \(\mu\)m. F, the scatter plot displays the association of TUNEL staining with \(trkC\) expression (as measured by Northern analysis). The average proportion of TUNEL positive nuclei in each tumor is highly correlated with expression of \(trkC\) (\(r = 0.8; P = 0.002\)). The proportion of apoptotic cells in 12 medulloblastoma samples was calculated by counting TUNEL-positive nuclei and dividing by the total number of nuclei (propidium iodide counterstained) in 10 high-powered fields (X40 objective) per tumor.
was correlated with apoptosis. Furthermore, proliferation as measured with the Ki-67 antibody did not correlate with trkC expression ($r = 0.21; P = 0.51$). These data from human tumors indicate that trkC overexpression is associated with apoptosis but not proliferation in primary medulloblastomas in vivo.

**DISCUSSION**

Although trkC expression serves as a marker for prognosis, our current data suggest a deterministic role for TrkC in medulloblastoma growth. We have observed that: (a) NT-3 stimulation influences tumor growth *in vitro* by inducing apoptosis, (b) spontaneous apoptosis is highly correlated with trkC-expression in primary medulloblastoma specimens, and (c) TrkC-expression inhibits the growth of intracerebral xenografts of the Daoy cell line medulloblastoma *in vivo*. We have detected *nt-3* transcripts in ~83% of patient tumor RNA specimens. Although the precise source of NT-3 has not been defined (Table 1), its coexpression with trkC suggests that endogenous activation of TrkC may regulate tumor growth.

We hypothesize that TrkC activity directly influences tumor growth by inducing apoptosis that promotes more favorable clinical outcomes. Because medulloblastomas do not spontaneously regress but continue to grow despite increased cell death in tumors with high trkC expression, the rate of apoptosis induced by activated TrkC presumably does not exceed the rate of tumor cell proliferation *in vivo*. Moreover, medulloblastomas are cured only in the context of treatment with surgery, radiation, and chemotherapy, indicating that the effects of TrkC activation act in concert with conventional cancer therapies in determining clinical outcome.

The growth of medulloblastomas, like many childhood tumors, reflects dysregulated responses to environmental cues and developmental signals such as neurotrophins. Most evidence available to date indicates that neurotrophins promote the differentiation or survival of cerebellar granule cells (12–15). Our data from the medulloblastoma cell line Daoy support the conclusion that NT-3-induced apoptosis of medulloblastomas occurs in the context of intact TrkC signaling. We have shown that NT-3 stimulation of the Daoy-trkC cell line activates multiple parallel signal transduction pathways involving PI3’K, ERK1/2, JNK/SAPK, and p38MAPK. Of these, p38MAPK activity appears necessary for NT-3/TrkC-induced apoptosis, based on experiments using pharmacological inhibitors. NT-3/TrkC-induced apoptosis also appears to require the expression of downstream target genes as shown by the protective effects of CHX. We have identified the IEGs c-jun and c-fos among the intermediates activated by TrkC signaling, which, in turn, may promote the expression of target genes responsible for apoptosis. Although our evidence argues that apoptosis in medulloblastomas occurs with appropriate TrkC signal transduction, this unusual response may arise because constitutively proliferating cancer cells are incapable of executing an appropriate differentiation program in an abnormal, transformed intracellular context.

A report by Muragaki *et al.* (47) supports the apoptotic function of neurotrophin/Trk signaling in medulloblastoma-derived cell lines (47). However, they reported that transfected medulloblastoma cell lines undergo apoptosis in response to TrkA activation, whereas those expressing TrkC undergo differentiation when grown in the presence of NT-3, similar to neuroblastomas with TrkA-activation (48). These differences probably arise from details in experimental design (such as our use of serum-free medium), or possibly from differences in the level of receptor protein expression or from clonal variation within cell lines. Regardless of the divergence of our results, the physiological relevance of these different studies lies in the common ability of Trk receptor kinase activity (of either TrkA or TrkC) to induce apoptosis in medulloblastomas, in contrast to their canonical survival-promoting effects in normal neurons. Neither morphological evidence of differentiation nor changes in marker expression were appreciated in our NT-3-stimulated Daoy-trkC cultures. Similarly, activation of growth factor receptors in other tumor cell lines, including neuronal and glial phenotypes, have been shown to induce or enhance apoptosis, independent of differentiation (49–52). Our experimental observations of NT-3-induced apoptosis in primary medulloblastoma cultures and our correlation of apoptosis with trkC expression in primary medulloblastomas are most consistent with the conclusion that apoptosis is the predominant biological response to TrkC activation in these poorly differentiated tumors.

Although our evidence implicates TrkC activation as a modulator of medulloblastoma growth and survival, other molecules must be considered as the initiators of tumorigenesis. One candidate primary defect in medulloblastoma oncogenesis is mutation of *PTCH*, the human homologue of the *Drosophila* segment polarity gene, patched, which has been identified as the genetic lesion in individuals with Gorlin’s syndrome (nevoid basal cell carcinoma syndrome), an autosomal dominant disorder associated with increased incidence of medulloblastomas through loss of heterozygosity in tumors (53–55). Site-directed mutation of *PTCH* promotes the development of medulloblastomas in mice (56). These data identify *PTCH* as a cerebellar tumor suppressor gene. Furthermore, the APC (adenomatous polyposis colii) gene has emerged as another candidate tumor suppressor gene in medulloblastoma oncogenesis (57).

Together with this report, the evidence to date implies that growth-promoting mutations of genes such as *PTCH* or *APC* act in combination with growth-inhibiting actions of TrkC to determine the behavior of medulloblastomas. This model of medulloblastoma progression is consistent with other studies, which reveal that apoptosis profoundly slows the growth of central nervous system tumors (58, 59). Further investigation will be required to explore the taming possibility of using physiological growth factors to enhance apoptosis in medulloblastomas without the short-term side effects or the long-term sequelae of conventional treatments.

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