Nerve Growth Factor Induces Apoptosis in Human Medulloblastoma Cell Lines that Express TrkA Receptors

Yoshihiro Muragaki,1,2 Thomas T. Chou,1 David R. Kaplan,3 John Q. Trojanowski,1 and Virginia M.-Y. Lee1

1Department of Pathology and Laboratory Medicine, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-4283, 2Department of Neurosurgery, Tokyo Women's Medical College, Tokyo, Japan, and 3The ABL-Basic Research Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702

Nerve growth factor (NGF) is the most well-studied representative of a family of trophic factors (neurotrophins), and NGF has pleiotropic effects including the ability to induce differentiation, support cell survival, and prevent apoptosis in neuronal progenitor cells and immature neurons, and other cells. Here, we examined the effects of nerve growth factor (NGF) and its cognate receptor (Trk or TrkA) on the survival of a common childhood brain tumor, i.e., medulloblastoma, a tumor that resembles CNS neuroepithelial progenitor cells. To do this, we engineered two human medulloblastoma cell lines (i.e., D283MED and DAOY cells) to express human TrkA using a retroviral expression vector. Surprisingly, NGF-treated medulloblastoma cells expressing the TrkA receptor (D283trk and DAOYtrk cells) grown in the presence or absence of serum underwent massive apoptosis, but similar treatment did not induce apoptosis in wild-type uninfected cells, cells expressing an empty vector, or cells expressing the TrkC receptor. Furthermore, D283MED cells engineered to express the human p75 NGF receptor (D283p75) also did not undergo apoptosis. Significantly, NGF-induced apoptosis in D283trk and DAOYtrk cells can be inhibited by anti-NGF antibodies and by K-252a, an inhibitor of TrkA tyrosine phosphorylation and mimicked by high concentrations of NT3. Because NGF treatment primarily eliminated D283trk cells from the S phase of the cell cycle, this form of NGF-mediated apoptosis is cell cycle-dependent. These findings suggest that a NGF/TrkA signal transduction pathway could activate apoptotic cell death programs in CNS neuroepithelial progenitor cells and in childhood brain tumors.

Key words: nerve growth factor; neurotrophins; medulloblastoma; TrkA; apoptosis; S phase

Nerve growth factor (NGF) act through their cognate receptors to promote the differentiation and/or survival of neuronal progenitor cells, immature neurons, and other cells. Here, we examined the effects of nerve growth factor (NGF) and its cognate receptor (Trk or TrkA) on the survival of a common childhood brain tumor, i.e., medulloblastoma, a tumor that resembles CNS neuroepithelial progenitor cells. To do this, we engineered two human medulloblastoma cell lines (i.e., D283MED and DAOY cells) to express human TrkA using a retroviral expression vector. Surprisingly, NGF-treated medulloblastoma cells expressing the TrkA receptor (D283trk and DAOYtrk cells) grown in the presence or absence of serum underwent massive apoptosis, but similar treatment did not induce apoptosis in wide-type uninfected cells, cells expressing an empty vector, or cells expressing the TrkC receptor. Furthermore, D283MED cells engineered to express the human p75 NGF receptor (D283p75) also did not undergo apoptosis. Significantly, NGF-induced apoptosis in D283trk and DAOYtrk cells can be inhibited by anti-NGF antibodies and by K-252a, an inhibitor of TrkA tyrosine phosphorylation and mimicked by high concentrations of NT3. Because NGF treatment primarily eliminated D283trk cells from the S phase of the cell cycle, this form of NGF-mediated apoptosis is cell cycle-dependent. These findings suggest that a NGF/TrkA signal transduction pathway could activate apoptotic cell death programs in CNS neuroepithelial progenitor cells and in childhood brain tumors.

Key words: nerve growth factor; neurotrophins; medulloblastoma; TrkA; apoptosis; S phase

Received Aug. 9, 1996; revised Oct. 17, 1996; accepted Oct. 23, 1996.

This work was supported in part by grants from National Institutes of Health and by a Zenith Award from the Alzheimer’s Association. We thank Mr. N. Timothy and Ms. S. Chiu for technical assistance, Drs. D. Bigner and H. Friedman for the D283MED cell line, Dr. J. M. Verdi for the pLVSHDtrk retroviral vector, Dr. J. Wolfe for the GPeVamp12 packaging cell line, Dr. L. A. Greene for the PC12med subline, Dr. M. V. Chao for NIH-3T3trk cells, and Dr. B. Hempstead for PC12-615 cells. We also thank Mr. J. C. Mills and Dr. R. Pittman for their comments on this paper and their assistance with Hoechst staining and videomicroscopy, and Drs. J. Peringa, S. Wang, and K.-M. Fung for help with methods to detect apoptosis.

Correspondence should be addressed to Virginia M.-Y. Lee, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, HUP, Makoney Building, Room A009, Philadelphia, PA 19104-4283.

Dr. Kaplan’s present address: Montreal Neurological Institute, 3801 University Street, Field House, Montreal, Quebec, Canada H3A 2B4.

Y.M. and T.T.C. contributed equally to this work.

Copyright © 1997 Society for Neuroscience 0270-6474/97/170530-13$05.00/0
neuronal lineage (e.g., D283MED) are thought to be more differentiated, whereas medulloblastoma cell lines that do not express markers of neuronal or glial lineage (e.g., DAOY) are thought to be more embryonal (Trojanowski et al., 1994; Perring et al., 1995). Because no medulloblastoma cell line expresses p75, we engineered one of these lines (i.e., D283MED or D283) to express human p75, but this cell line (known as A009 or D283p75) did not differentiate or cease dividing in response to treatment with exogenous NGF (Pleasure et al., 1990). Thus, we hypothesize that the TrkA receptor may be required to mediate responses to NGF in medulloblastoma cells. To test this hypothesis, we engineered the more differentiated D283 cells and the less differentiated DAOY cells to express TrkA on their cell surface by infecting these cells with a retrovirus harboring human TrkA, and then exposing these infected cells (D283trk and DAOYtrk cells) to exogenous NGF. Here, we show that D283trk and DAOYtrk cells respond to NGF by undergoing massive apoptosis.

**MATERIALS AND METHODS**

*Tissue culture, retroviral infection, and NGF treatment*

Uninfected D283MED (D283) and DAOY cells and cells infected with retrovirus were maintained with RPMI 1640 medium containing 10% fetal bovine serum and 2 mM glutamine (Friedman et al., 1985; Trojanowski et al., 1989). DAOY cells normally grow in a monolayer, whereas the D283 cells grow in suspension. Thus, for the MTs assays, fluorescence, and videomicroscopy as well as for the immunoblotting and immunostaining, monolayer cultures of the D283 cells were established by plating the cells onto poly-D-lysine-coated (1 μg/ml) tissue culture dishes or coverslips as described previously (Pleasure et al., 1990). For studies involving the use of serum-free medium, fetal bovine serum was omitted from the culture medium.

Retroviruses bearing a full-length human *trkA* cDNA (*trkA* isoform; Barker et al., 1993) cloned into the retroviral vectors (Miller and Kosman, 1989) pLNCX (designated as pLNCtrk; Stephens et al., 1994) and pLHDCX (designated as pLHDCtrk; Verdi et al., 1994) were packaged by electroporation in the GPenvamp12 packaging cell line (Markowitz et al., 1988). Supernatants containing the retroviruses were harvested and used directly to infect the medulloblastoma cell lines. After 16 hr of infection, the viral supernatants were removed and the cells were incubated with media alone for an additional 24 hr before selection with media alone for an additional 24 hr before selection with the pLNCtrk constructor with L-histidinol (Sigma, St. Louis, MO) at 8 μM for the pLHDCtrk construct for an additional 3 weeks. The drug-resistant DAOY cells (designated as D283Ntrk and D283Htrk cells, respectively) were used either as a mass culture, or they were subcloned by limited dilution to obtain clonal lines that express high levels of *trkA*. Similar responses to NGF were obtained with the mass cultures and with the mass cultures and with the mass cultures and subclones of the D283trk and the D283Htrk cells (data not shown). As controls, D283 cells were also infected with a retrovirus bearing the pLNCX empty retroviral vector (D283vec cells) or infected with a retrovirus containing human p75 (designated as D283p75 cells; Pleasure et al., 1990) or with the pLNCtrkC construct. The pLNCtrk construct was also used to infect DAOY, PC12, and NIH-3T3 cells to generate a stable population of cells expressing the TrkA receptor. Additionally, the pLNCtrkC construct was also used to infect DAOY, PC12, and D283 cells to generate stable populations of cells that expressed the human TrkC receptor.

Northern blots, immunoblots, and indirect immunofluorescence

Total RNA was extracted with Trizol (World Precision Instruments), and RNA (25 μg) was then electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, followed by transfer of the separated RNAs to a nylon membrane. After drying the membranes, the immobilized RNAs were probed with a full-length human *trkA* cDNA probe labeled with [32P]CTP for 3 hr and washed twice with 2 × SSC + 0.1% SDS at 42°C, twice with 0.2 × SSC + 0.1% SDS at 42°C, and once with 0.1 × SSC + 0.1% SDS at 55°C. The labeled RNA species in these blots were visualized using a Phosphoimager and analyzed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Measurement of cell viability**

Cell viability was quantitated using the MTS (3-(4,5-dimethythiazol-2-yl)-(3-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay according to procedures recommended by the vendor (Promega, Madison, WI). To do this, 5 × 10^4 cells in serum-containing medium or 1 × 10^5 cells in serum-free medium were seeded into 96 wells of 96-well plates after incubating wild-type and genetically modified cells for 4 d in NGF as described above. The absorbance values at 450 nm were quantitated and compared with the absorbance values obtained from wells containing wild-type or genetically modified sources of NGF were also used. Mouse NGF (2.5S) was obtained from Collaborative Biomedical Products (Bedford, MA), and human recombinant NGF-β was obtained from Sigma. All NGF preparations were used to examine the biological effects of NGF on the wild-type and the genetically modified medulloblastoma cell lines (i.e., D283 and DAOY). The specificity of these effects was monitored by using a rabbit antibody to 2.5S mouse NGF (Sigma) as well as a monoclonal antibody to recombinant NGF (Boehringer Mannheim, Indianapolis, IN) in blocking experiments. Recombinant NT3 was a gift from Regeneron (Tarrytown, NY).
D283 cells that were not treated with NGF. Each MTS assay was repeated at least three times, and each experiment was done in triplicate. Furthermore, the results of the MTS assays correlated well with cell counts obtained using trypan blue exclusion assay.

**Measurements of apoptotic cell death**
Three complementary methods were used to monitor apoptotic cell death in NGF-treated Trk expressing medulloblastoma cells.

- **Hoechst 33342 staining of apoptotic bodies.** In this assay, cells were grown in suspension, incubated with 100 ng/ml of NGF for 4 d, and stained with 5 μg/ml of the Hoechst 33342 dye for 5 min at room temperature. Normal nuclei and nuclear apoptotic bodies stained by the Hoechst 33342 dye were then visualized and monitored in each set of experiments by fluorescence microscopy.

- **Detection of DNA fragmentation induced by apoptosis.** To detect DNA “laddering” in cells undergoing apoptosis, wild-type and genetically modified D283 cells were incubated with 100 ng/ml of NGF for 3 d, and DNA from ~8 × 10^6 cells was extracted as described (Tilly and Hsu, 1993). The DNA was electrophoresed in 2% agarose gels, stained with 2 μg/ml ethidium bromide for 30 min at room temperature, and destained using distilled water at 4°C overnight. Alternatively, DNA “laddering” was detected in gels of similar DNA extracts by labeling the DNA fragments with [32P]dATP (30 μCi) and TdT (25 U) at 37°C for 1 h before electrophoresis. In this procedure, unincorporated nucleotides were separated from the labeled DNA by precipitating the DNA twice with ethanol. These DNA samples were electrophoresed in 2% agarose gel, and the gel was denatured with 1.5 M NaCl/0.5 M NaOH for 30 min followed by an extensive wash with 1.5 M NaCl/0.1 M Tris, pH 7.0, for 30 min. After air-drying, these membranes were then analyzed with a Phosphoimager as described above.

- **Visualization of apoptosis by time-lapse videomicroscopy.** Living wild-type and genetically modified D283 cells treated with NGF were monitored for evidence of apoptosis (e.g., membrane ruffling and blebbing, formation of apoptotic bodies) by videomicroscopy for up to 4 d during treatment with NGF as described (Pittman et al., 1993; Mills et al., 1995).

**Flow cytometric analysis of apoptosis during different phases of the cell cycle**
To monitor the occurrence of apoptosis during different phases of the cell cycle by flow cytometry, ~2.5 × 10^6 of the D283trk cells were incubated with NGF for different lengths of time, stained with 50 μg/ml propidium iodide in hypotonic buffer containing 0.1% Triton X-100 and 0.1% sodium citrate at 4°C overnight (Nicoletti et al., 1991), and analyzed with a FACScan (Becton-Dickinson Immunocytochemistry Systems, Mountain View, CA). The stained cells traversed the beam of light (488 nm wavelength) emitted by an argon laser. A 560 nm dichroic mirror and a 585 ± 21 nm bandpass filter were used for data collection. The forward scatter and side scatter of the red fluorescent particles (resulting from propidium iodide-stained DNA) were measured simultaneously with a FACScan (Becton-Dickinson Immunocytochemistry Systems, Mountain View, CA). The DNA was electrophoresed in 2% agarose gels, stained by 2 μg/ml ethidium bromide, and visualized by fluorescence microscopy (resulting from propidium iodide-stained DNA). The measurements of DNA content were made by counting the number of DNA stained with 5 μg/ml of Hoechst 33342 dye were then visualized and monitored in each set of experiments by fluorescence microscopy.

- **Hoechst 33342 staining of apoptotic bodies.** In this assay, cells were grown in suspension, incubated with 100 ng/ml of NGF for 4 d, and stained with ~8 × 10^6 cells as described (Tilly and Hsu, 1993). The DNA was electrophoresed in 2% agarose gels, stained with 2 μg/ml ethidium bromide for 30 min at room temperature, and destained using distilled water at 4°C overnight. Alternatively, DNA “laddering” was detected in gels of similar DNA extracts by labeling the DNA fragments with [32P]dATP (30 μCi) and TdT (25 U) at 37°C for 1 h before electrophoresis. In this procedure, unincorporated nucleotides were separated from the labeled DNA by precipitating the DNA twice with ethanol. These DNA samples were electrophoresed in 2% agarose gel, and the gel was denatured with 1.5 M NaCl/0.5 M NaOH for 30 min followed by an extensive wash with 1.5 M NaCl/0.1 M Tris, pH 7.0, for 30 min. After air-drying, these membranes were then analyzed with a Phosphoimager as described above.

**Western blot analysis using E7, a mAb that recognizes an epitope in the extracellular domain of human TrkA (Fig. 1B).** These studies showed that both the D283Ntrk and DAOYtrk cell lines exhibited two distinct TrkA immunoreactive bands of ~125 and 110 kDa. The poorly or nonglycosylated 110 kDa TrkA band migrated similarly in the PC12trk, D283trk, and DAOYtrk cells. However, the higher Mr 125 kDa TrkA band in both D283trk and DAOYtrk cells showed a more rapid electrophoretic mobility than the 140 kDa TrkA band in the 3T3trk and PC12trk cells, which suggests that this NGF receptor may not be glycosylated as extensively in the D283trk and the DAOYtrk cells as it is in the 3T3trk and PC12trk cells. It is evident that neither wild-type D283 nor DAOY contain detectable amounts of TrkA. Furthermore, the expression levels of TrkA in the D283trk cells were quite a bit lower than in the 3T3trk and PC12trk cells, whereas the expression levels of TrkA in DAOYtrk cells were comparable with those in the 3T3trk and PC12trk cells (Fig. 1B). Because the D283trk cell line was used for all subsequent experiments, we refer to these cells as the D283trk cell line hereafter for simplicity. In addition, to confirm and extend the results obtained with the D283trk cells, we conducted selected studies on the DAOYtrk cells in parallel with those performed on the D283trk cell line, and representative data from these studies are reported below.

To determine whether TrkA was expressed on the plasma membrane of the D283trk cells, indirect immunofluorescence was performed on live D283trk cells using E13, a mAb that binds to an epitope in the extracellular domain of TrkA (Muragaki et al., 1995). These studies demonstrated that TrkA receptor proteins were expressed on the surface plasma membrane of the D283trk cells, whereas no immunoreactive TrkA was detected on the surface of the control D283vec cells (compare Fig. 2A, a with b). Similarly, TrkA receptor proteins also were expressed on the cell surface of the DAOYtrk cells but not on wild-type DAOY cells (compare Fig. 2A, c with d). To determine whether TrkA became activated after treatment of the D283trk cells with NGF, we monitored the ability of TrkA receptor proteins to undergo NGF-induced autophosphorylation on tyrosine residues. This was accomplished by treating the D283trk cells for 5 min with NGF followed by immunoblotting from cell lysates with an anti-phosphotyrosine antibody. These studies showed that in the absence of NGF, there was little or no autophosphorylation of the TrkA receptors that were expressed in the D283trk cells (Fig. 2B).
However, brief exposure of these cells to NGF induced autophosphorylation of tyrosine residues in TrkA. Finally, we confirmed that the immunoband detected by the anti-phosphotyrosine antibody was TrkA by stripping the nitrocellulose replicas and reprobing them with the E7 mAb, which detected the same TrkA immunoband in cell lysates prepared from NGF-treated or untreated cells (Fig. 2B).

**NGF induces cell death in medulloblastoma cells engineered to express TrkA**

To determine the nature of the biological response of the Trk expressing medulloblastoma cells to NGF, we treated similar aliquots of the different retrovirally infected medulloblastoma cells described above with NGF. The response of these cell lines to NGF was then monitored using a number of different complementary assays of cell number and viability (Figs. 3–6). After a 3 d incubation with 100 ng/ml of NGF in complete culture medium (i.e., containing 10% fetal bovine serum), the wells plated with D283trk cells contained fewer cells than the wells containing the D283vec and D283p75 cells (compare Fig. 3, a with b, c with d, and e with f). Similarly, the DAOYtrk cells also contained fewer cells after treatment with NGF (Fig. 3g). Furthermore, a large number of the D283trk and DAOYtrk cells were rounded up and floating, which suggested that they had undergone cell death. Notably, this effect of NGF was observed regardless of whether the D283trk cells were grown in suspension or in monolayer culture on poly-D-lysine-coated dishes. Additionally, a similar response to NGF was seen after stable infection of D283 cells with either the pLNCtrk or the pLHDCtrk vector as well as in sub-

---

**Figure 1.** A, Expression of *trkA* transcripts in D283 cells infected with *trkA*-containing retroviral vectors. Northern blots were performed on 25 μg of total RNA from different cell lines following electrophoresis using cDNA probes to full-length human *trkA*. Notably, both the D283trk cells infected with pLNCtrk (D283Ntrk, lane 7) and with pLNHDtrk (D283Htrk, lane 8) retroviral vectors expressed high levels of *trkA* transcripts. In contrast, no *trkA* transcripts were seen in the parent D283 cells (lane 4), D283 cells infected with empty vector (D283vec, lane 5), or in the D283 cells infected with a retrovirus containing human p75NGFR (D283p75, lane 6). Note that the rodent phaeochromocytoma-derived PC12 cells express an ~3.2 kb transcript corresponding to rat *trkA* (asterisk). The two bands in D283trk cells correspond to *trkA* transcripts situated between the 5' and 3' LTRs of the retroviral vector (top bands) and between the internal CMV promoter and 3' LTR of this vector (bottom bands). The 28S and 18S molecular weight markers are shown to the left of lane 1 in the Northern blot, and the ethidium bromide-stained gel below demonstrates equal loading of the 28S and 18S ribosomal RNAs from the cell lines in each of the lanes shown above in the Northern blot. B, TrkA receptor protein expression in different cell lines engineered stably to express TrkA. Western blots were performed on cell extracts (100 μg of protein per lane) from each of the cell lines indicated above each lane in B after electrophoresis in 7.5% SDS-PAGE gels. TrkA was detected in blots probed with E7, a MAb directed against the extracellular domain of TrkA. The ascites was applied at a dilution of 1:1000. Both the D283trk (lane 3) and the DAOYtrk (lane 4) cell lines express ~110 and ~125 kDa (short arrow) TrkA immunobands, both of which migrate more rapidly than the 140 kDa (long arrow) species of fully glycosylated TrkA observed in the PC12trk (lane 5), 3T3trk cells (lane 6). Furthermore, trkC expressed in DAOYtrkC cells almost comigrated with TrkA bands from 3T3trk cells (compare lanes 6 and 7). No immunoreactive TrkA is seen in the wild-type D283 and DAOY cells (lane 1 and lane 2, respectively).
clones of D283trk and DAOYtrk cells that expressed different levels of TrkA receptor protein (data not shown).

To demonstrate that the reduction in the number of D283trk and DAOYtrk cells was specific to NGF treatment, we conducted several sets of control experiments. First, we infected PC12mut (a PC12 mutant that does not express detectable TrkA and, therefore, does not respond to NGF) with the same trkA retroviral construct (i.e., pLNCtrk) and showed that these cells expressed functional TrkA receptors by extending long processes in response to treatment with NGF (data not shown). Second, because the NGF used in the experiments described above was prepared in our laboratory from extracts of salivary glands harvested from male mice, as described earlier (Mobley et al., 1976), we also used commercially available 2.5S mouse NGF (Collaborative Research) and recombinant NGF (Sigma) to demonstrate that NGF from both commercial sources had similar effects on the D283trk
and DAOYtrk cells (data not shown). A third control was performed by adding an anti-NGF antibody to the culture medium, which blocked the effects of NGF treatment on the D283trk cells in a dose-dependent manner. For example, at an antibody dilution of \( \leq 1:500 \) of a polyclonal antiserum, anti-NGF antibody completely blocked the effects of NGF on the D283trk cells (compare Fig. 4, a with b). Similar effects also were observed using a mAb to NGF (data not shown). Fourth, pretreatment of D283trk and DAOYtrk cells with K-252a, a specific blocker of TrkA tyrosine phosphorylation (Berg et al., 1992), completely blocked the induction of cell death by NGF (Fig. 4c,d). Fifth, additional controls were performed using NT3 (i.e., the neurotrophin that is the cognate ligand for the TrkC receptor), because NT3 also binds weakly to TrkA receptors (Cordon-Cardo et al., 1991; Ip et al., 1993; Clary and Reichardt, 1994). These experiments showed that treatment of the D283trk and DAOYtrk cells with 100 ng/ml NT3 only had a small effect on the number of D283trk and DAOYtrk cells. However, at 1 \( \mu \text{g/ml} \) and higher concentrations, NT3 also induced cell death in TrkA expressing D283 and DAOY cells (compare Fig. 4, e with f). Finally, as additional controls, we treated D283 and DAOY cells that were engineered to stably express the TrkC receptor with NT3, but this did not augment or enhance the death of cells in either of these cell lines (Fig. 4g,h). Thus, this set of experiments provides additional evidence indicating that the effects of NGF treatment on the number of TrkA-expressing medulloblastoma cells is specific to NGF.

To quantify the reduction in the number of NGF-treated D283trk cells, we monitored the viability of D283trk cells using the MTS assay that was performed on cells treated with different concentrations of NGF. These experiments were conducted after standard curves for the MTS assay were established to document a linear relationship between the number of D283trk cells and the absorbance values of MTS at 450 nm. After treatment of the D283trk cells with NGF for 4 d in complete medium, the MTS assay confirmed that there was a dramatic reduction in the number of viable D283trk cells (Fig. 5A). Moreover, an inverse dose-
dependent relationship was seen between the concentration of NGF and the number of remaining D283trk cells. Because 50% of the D283trk cells were eliminated by treatment of these cell lines with 1–5 ng/ml NGF, it is likely that this effect of NGF is mediated by the high-affinity TrkA receptors. This interpretation was supported by parallel studies of the D283vec and D283p75 cell lines, because neither of these cell lines showed any significant reduction in cell viability (as monitored by the MTS assay) after treatment with NGF (Fig. 5A). To determine whether serum was required for the induction of cell death in the D283trk cells by treatment with NGF, we also performed the MTS assay on cells cultured in serum-free medium (Fig. 5B). In these experiments, NGF caused a 51% reduction in the viability of the D283trk cells at 100 ng/ml compared with D283trk cells that were not treated with NGF. Although the D283vec cells did not show any changes in their response to treatment with NGF under similar conditions, NGF treatment did have a mild effect on the viability of the D283p75 cells, and this effect was significant after treatment with 10 ng/ml NGF (Fig. 5B). Taken together, these findings demonstrate that NGF selectively compromises the viability of D283trk cells and that the ability of NGF to induce cell death in the D283trk cells is independent of the presence or absence of fetal bovine serum in the culture medium.

NGF-induced cell death in D283trk and DAOYtrk cells exhibits hallmarks of apoptosis

To characterize the type of cell death induced by NGF in the D283trk cells, we used three different complementary strategies: (1) fluorescence microscopy and staining with Hoechst 33342 dye, (2) gel electrophoresis to detect DNA fragmentation, and (3) time-lapse videomicroscopy. In addition, NGF-treated DAOYtrk cells also were stained with Hoechst 33342 dye and examined by fluorescence microscopy to determine whether they also showed morphological evidence of apoptosis.

Immunofluorescence studies performed on cells stained with the Hoechst 33342 dye revealed condensation and fragmentation of the nuclear chromatin in numerous D283trk cells treated with NGF for 4 d, whereas similar nuclear changes were extremely rare or absent in untreated D283trk cells as well as in the NGF-treated D283vec cells (Fig. 6A, a–c). Similarly, when DAOYtrk cells were...
Because videomicroscopy enables prolonged in vitro observations of individual cells after experimental manipulation, we examined the D283trk cells by this method after NGF treatment. These videomicroscopy studies showed that many of the NGF-treated D283trk cells became spherical at 12 hr, followed by membrane ruffling and blebbing during the next 24–72 hr (Fig. 7). For example, Figure 7 illustrates representative serial changes in one of the NGF-treated D283trk cells during late stages of apoptosis, including the typical membrane blebbing associated with apoptosis followed by a loss of cell volume and disintegration of the cell. Because these morphological features are associated with active apoptosis (Deckwerth and Johnson, 1993; Pittman et al., 1993; Schiffer et al., 1994; Mills et al., 1995), the videomicroscopy observations provide additional evidence that NGF induces an apoptotic form of cell death in the D283trk cells. Most of the D283trk cells monitored by videomicroscopy died after treatment with NGF for 4 d, and many of these cells exhibited the morphological features of apoptosis described above and illustrated in Figure 7.

**NGF-induced apoptosis in the D283trk cell line is cell cycle-dependent**

Flow cytometric analysis was performed on NGF-treated D283trk cells to determine whether NGF induced apoptosis in a cell cycle-dependent manner. NGF treatment resulted in an ~70% decrease in the percentage of D283trk cells in the S phase of the cell cycle at 24 hr after treatment, with a more modest decrease in the number of these cells in the G0/G1 and G2/M phases of the cell cycle (Fig. 8A–C). Although the percentage of D283trk cells in G0/G1 and G2/M gradually decreased further with time (Fig. 8A–C), these studies demonstrate an early vulnerability of the S phase D283trk cells to apoptosis after treatment with NGF. The quantitative analysis of the absolute number of NGF-treated D283trk cells in each phase of the cell cycle demonstrated a similar preferential loss of S phase D283trk cells. For example, the number of D283trk cells in S phase promptly decreased by 70% within the first 24 hr of NGF treatment, whereas cells in the G2/M phase decreased much more gradually, i.e., by 46% after 96 hr of treatment with NGF.

**DISCUSSION**

The series of studies described here provide the first compelling evidence that NGF can act through the TrkA receptor to induce an apoptotic form of cell death in authentic human brain tumor-derived cell lines. Although histochecmical evidence of apoptosis has been observed in biopsy samples of human medulloblastomas (Schiffer et al., 1994), this is the first study to demonstrate directly that NGF specifically induces apoptosis in two human medulloblastoma-derived cell lines by activating its cognate high-affinity receptor, i.e., TrkA. Although NGF and other neurotrophins (i.e., BDNF, NT3, NT4/5, NT6) are known to have pleiotrophic effects on different cell types, these factors are primarily known for their ability to promote the proliferation, survival, and maturation of target cells, whereas cell death is generally thought to be a consequence of neurotrophin withdrawal rather than the exposure of cells to one of these factors (Deckwerth and Johnson, 1993; Pittman et al., 1993; Freeman et al., 1994; Barbacid, 1995; Mills et al., 1995). In addition, although each of the major neurotrophin receptors (including p75) as well as several neurotrophic factors have been detected in biopsy samples of human medulloblastomas (Baker et al., 1991; Segal et al., 1994; Washiyama et al., 1996) and preliminary studies of medulloblastoma...
Figure 6. A, Morphological evidence of apoptotic death in D283trk and DAOYtrk cells induced by treatment with NGF for 4 and 2 d, respectively. Hoechst 33342 staining did not demonstrate the formation of condensed chromatin in the D283vec cells treated with NGF (D283vec + NGF in a) or in the D283trk and DAOYtrk cells in the absence of NGF (b and d, respectively). However, condensed chromatin was seen in (Figure legend continues)
biopsies suggest that the levels of trkC mRNA correlate with a better response to therapy (Segal et al., 1994), it is unclear what effects NGF or other neurotrophins have on the biology of these or other human brain tumors. Hence, the studies described here have important implications for understanding the function of NGF and TrkA in the developing normal nervous system as well as in human brain tumors.

To elucidate the role of NGF and TrkA in the pathobiology of medulloblastomas, we generated the D283trk and DAOYtrk cell lines. We demonstrated that these cells expressed functional TrkA receptors on their plasma membranes, as evidenced by the ability of the TrkA receptors to undergo autophosphorylation at specific tyrosine residues in response to treatment with NGF. We then showed that NGF selectively and specifically induced an apoptotic form of cell death in the D283trk and DAOYtrk cells by using several different measures of cell viability and by applying several different morphological, histochemical, and biochemical criteria for the recognition of apoptosis including the presence of apoptotic bodies, blebbing of the cell membrane, the condensation and fragmentation of nuclear chromatin, and DNA laddering. Additionally, we confirmed that this apoptotic cell death response to NGF treatment was independent of factors present in serum, because NGF-treated D283trk cells underwent apoptosis when grown in either serum-containing or serum-free medium. Furthermore, we also presented data to suggest that NGF-induced apoptosis in the D283trk and DAOYtrk cells was mediated by the TrkA receptor. For example, D283 cells, engineered to express p75 (D283p75 cells), did not undergo apoptosis in response to NGF (Pleasure et al., 1990), and the half-maximal response of the D283trk cells to NGF only required 1–5 ng/ml NGF, which is indicative of high-affinity ligand/receptor binding. Additionally, several batches of mass cultures or clonally derived D283trk and DAOYtrk cells stably expressing varying levels of TrkA protein responded similarly to NGF. Other evidence also supports the notion that the induction of cell death in D283trk cells is specific to NGF, because NGF-induced apoptosis was blocked by both polyclonal and mAb to NGF, and at least two different preparations of NGF (including recombinant NGF) induced apoptosis in these cells. Notably, another member of the neurotrophin family that binds very weakly to the TrkA receptor (i.e., NT3) induced apoptosis in the D283trk and DAOYtrk cells but only at high concentrations. Furthermore, K-252a (a specific inhibitor of Trk tyrosine phosphorylation) completely blocked the effects of NGF. Finally, we also showed that NGF-induced apoptosis is specific to NGF and TrkA, because treatment of D283 and DAOY infected by the same retroviral vector bearing the TrkC receptors with NT3 did not result in cell death.

Although the studies summarized here provide compelling evidence that apoptosis is a consequence of the activation of NGF/TrkA signaling pathways, the induction of apoptosis in response to NGF treatment via the TrkA receptor is cell type-specific, because the expression of TrkA receptors in both the NIH-3T3 and PC12 cells using the same retroviral vector system did not lead to apoptosis after treatment of these cells with NGF.

Approximately 30 min before the death of this cell, a number of apoptotic bodies can be observed. Finally, more massive blebs develop (triangles in d1 and d2), and the cell body begins to disintegrate (d2).

**Figure 7.** Time-lapse videomicroscopy of apoptotic cell death in the D283trk cells after treatment with NGF. Cells (5 × 10^5) were plated on 35 mm coverglasses coated with 1 μg/ml poly-D-lysine and observed by time-lapse videomicroscopy to monitor cell death events at the times indicated in each image. Apoptosis was common, and a cell that died an apoptotic death beginning at ~57 hr after NGF treatment is followed at subsequent time intervals from 0 to 48 min in the images in all through d2. Initially, this cell appeared normal (a0), but typical blebs (arrows in b1 through b4) begin to develop in the NGF-treated D283trk cells. Approximately 30 min before the death of this cell, a number of apoptotic bodies can be observed. Finally, more massive blebs develop (triangles in d1 and d2), and the cell body begins to disintegrate (d2).
activation of Ras (Li et al., 1992; Thomas et al., 1992; Wood et al., 1992). Accordingly, because mice deficient in rasGAP, a negative regulator of Ras, display a striking increase in the apoptotic death of cells of the anterior neural tube and cranial neural crest (Henkemeyer et al., 1995), it will be important to determine whether apoptosis in TrkA expressing medulloblastoma cells also is mediated by Ras activation. Alternatively, it is possible that other kinases or molecules involved in some other signaling pathways also could play a role in the induction of apoptosis by NGF in medulloblastoma cells. For example, a recent study demonstrated that apoptosis induced by NGF withdrawal in NGF-dependent PC12 cells could be mediated by the activation of JNK (c-JUN NH2-terminal protein kinase) and p38 as well as by the concurrent inhibition of extracellular signal-regulated kinase (ERK) signaling pathways (Xia et al., 1995). It will be informative to determine whether downstream events leading to apoptosis in NGF-treated TrkA-expressing medulloblastoma cells also might be mediated by similar signaling pathways.

Our finding that NGF selectively reduces the percentage of D283trk cells in S phase agrees with previous reports that suggest a tight association between DNA synthesis and apoptosis (Qin et al., 1994; Shan and Lee, 1994). In this respect, a conflict in growth and differentiating signals have been postulated to induce inappropriate cell cycle genes that might launch a cascade of events leading to apoptosis (Freeman et al., 1994). Nonetheless, the cell cycle-specific effects of NGF are far from understood at this time. Because our data clearly show that NGF induces autophosphorylation of the TrkA receptor in D283trk cells, it is likely that this initial step is shared by signaling pathways leading to cell death as well as to cell survival and differentiation.

Our studies may have important implications for understanding normal development and tumor progression. For example, the induction of apoptosis through activation of the TrkA receptor in TrkA-expressing medulloblastoma cells suggests a novel signaling mechanism that might play a role in normal developmentally regulated programmed cell death, because medulloblastomas resemble embryonic neuroectodermal stem cells or their immature neuronal and glial progeny (Molenaar et al., 1989; Gould et al., 1990; Trojanowski et al., 1994). On the other hand, if the findings reported here reflect the response of TrkA expressing medulloblastoma cells in vivo to NGF, then activation of NGF/TrkA signaling pathways in these tumors may regulate their growth and expansion by inducing apoptosis. Thus, additional studies of these signaling pathways may provide insights into mechanisms that regulate the normal development of the nervous system as well as the induction and progression of medulloblastomas and other pediatric brain tumors.

REFERENCES


