A Novel Apoptotic Pathway Induced by Nerve Growth Factor-mediated TrkA Activation in Medulloblastoma*

(Received for publication, June 8, 1999, and in revised form, October 21, 1999)

Thomas T. Chou, John Q. Trojanowski, and Virginia M.-Y. Lee‡

From the Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Nerve growth factor (NGF) induces apoptosis in a human medulloblastoma cell line (MED283) engineered to express TrkA (MED283-TrkA) (Muragaki, Y., Chou, T. T., Kaplan, D. R., Trojanowski, J. Q., and Lee, V. M. (1997) J. Neurosci. 17, 530–542) to dissect the molecular signaling pathway that mediates this novel effect, specific receptor mutations in Trk have been employed. We showed that phosphorylation of tyrosine 490 is required for activation of phosphoinositide 3-0H kinase, whereas phosphorylation of tyrosine 785 is required for activation of phospholipase C-γ. TrkA-mediated apoptosis was abolished when either the ATP-binding site or both tyrosines 490 and 785 were mutated. Because tyrosines 490 and 785 mediate redundant signaling through the Ras-extracellular signal-regulated kinase (Ras-ERK) pathway, we examined the role of Ras-ERK signaling in NGF-induced apoptosis. We found that MED283-TrkA cells expressing a dominant negative Ras inhibitor (N17Ras) failed to undergo ERK activation and apoptosis following NGF treatment, whereas the ERK kinase (mitogen-activated protein kinase kinase) inhibitors PD98059 and U0126 eliminated ERK activation but had no effect on apoptosis. We infer from these data that NGF-induced apoptosis is mediated by a novel Ras and/or Raf signaling pathway.

NGF plays a critical role in the development of the nervous system by regulating the proliferation, differentiation, and survival of neurons and their precursors (1). The signal transduction mechanism by which NGF generates diverse cellular responses has been studied extensively in vitro using a variety of cell culture model systems, most notably the rat pheochromocytoma cell line PC12 (2). The effects of NGF on PC12 cells are mediated by the high affinity NGF receptor known as TrkA, which is one of the three major cognate receptors of neurotrophins (3, 4). NGF activates TrkA through receptor dimerization and activation of an intrinsic tyrosine kinase (5). Trans-phosphorylation of TrkA by its intrinsic kinase stabilizes the receptor and facilitates phosphorylation of recognition sites for associated signaling molecules (6). For example, among the better characterized proteins that associate with phosphorylated TrkA are SHC and PLC-γ, which bind to tyrosine residues at positions 490 and 785, respectively (7, 8). Both of these proteins mediate signaling through the well characterized Ras-ERK pathway and ultimately affect changes in gene expression (9, 10). SHC is also critical for the activation of phosphotyrosin-3-0H kinase (11), which, along with its substrate Akt, participates in the prevention of apoptosis in PC12 cells following NGF withdrawal (12, 13).

Whereas in PC12 cells NGF promotes cell cycle arrest, differentiation, and survival, NGF mediates pleiotropic effects that are cell type-specific. For example, chromaffin cells and TrkA-expressing NIH-3T3 fibroblasts proliferate in response to NGF (14, 15). The mechanism whereby NGF induces differentiation in one cell type and proliferation in another remains unclear, but NGF-mediated differentiation has been linked to at least two specific protein kinase targets. First, fibroblast growth factor receptor substrate-2, a lipid-anchored docking protein of tyrosine kinase receptors, is preferentially tyrosine-phosphorylated during NGF-induced differentiation (16, 17). And second, the cyclin-dependent-kinase inhibitor p21Waf1/Cip1 is selectively induced in cells that undergo cell cycle arrest in response to NGF (17, 18). These observations suggest that the cell type-specific effects of NGF may be determined by the activation of distinctly different signaling pathways in cells of different lineages.

Although there is an extensive body of literature on the important role of neurotrophins in the developing and mature nervous system, only recently have neurotrophins been implicated in the onset or progression of central nervous system tumors. For example, NGF has been suggested to influence the phenotype of MBs, i.e. prototypical primitive neuroectodermal tumors that arise in the cerebellum and that are among the most common central nervous system tumors in children (19). Morphologically, MBs resemble neuroectodermal stem cells, and they often show a neuron-like phenotype, as evidenced by the expression of neuron-specific lineage markers (20). MBs are thought to arise from granule cell progenitors in the external granule layer of the developing cerebellum (21), and it has been reported that the abundance of trkC mRNA expression correlates with a better response to therapy in pediatric patients with MB (22). Although our earlier studies failed to implicate NGF and low affinity NGF receptor in modulating the behavior of MB cells (23), a subsequent study of the same MB cells engineered to express TrkA on their cell surface yielded the unexpected finding that activation of these receptors led to extensive apoptosis (24). We hypothesized that NGF may induce apoptosis specifically through activation of specific signal transduction pathways. To begin to elucidate this pathway, we undertook studies described herein, which demonstrate that

* This work was supported in part by grants from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Center for Neurodegenerative Disease Research, 3rd Floor Maloney Bldg., 56th & Spruce St., Philadelphia, PA 19104–4283. Tel.: 215–662–6920; Fax: 215–349–5909; E-mail: vmylee@mail.med.upenn.edu.

† The abbreviations used are: NGF, nerve growth factor; BAF, boc-aspartyl (O Me)-fluoromethylketone; ERK, extracellular signal-regulated kinase; GFP, green fluorescence protein; MB, medulloblastoma; MEK, mitogen-activated protein kinase kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium; PLC-γ, phospholipase C-γ.
NGF induces apoptosis in TrkA-transfected MED283 cells (MED283-TrkA) via redundant pathways that are Ras-dependent but MEK-independent. Thus, we report the initial molecular dissection of a novel cell death pathway in MB and suggest that further dissection of this cell death pathway may provide new targets for the development of more effective therapies for these and other brain tumors.

EXPERIMENTAL PROCEDURES

Materials—Murine NGF was extracted and purified from mouse salivary glands as described previously (25). NGF was used at a final concentration of 100 ng/ml. Cytokine growth medium was purchased from Sigma. K-252a was a gift from Kamiya Biochemical. BAF was purchased from Enzyme Systems Products. PD98059 was purchased from New England Biolabs. U0126 was purchased from Promega. The anti-Trk (E7) monoclonal antibody Y13–259 identified recombinant viruses. The anti-α-tubulin (DM 1A) monoclonal antibody was purchased from Sigma. The anti-Ras (Y13–259) monoclonal antibody was purchased from American Type Culture Collection. The anti-ERK (E16) monoclonal antibody and the anti-phosphoglycerogen synthase kinase-3 polyclonal antibody were purchased from New England Biolabs.

Cell Culture—The human medulloblastoma cell line MED283 was grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) (27). PC12–615 cells, derived from PC12 cells stably transfected to overexpress trk by 15–20-fold (28), were grown in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, and antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mg/ml G418) (29).

Retroviral Transfection—Retroviral vectors pLNCX carrying human TrkA wild-type, TrkA Y490F, TrkA Y785F, TrkA YY490/785F, and TrkA K535N, and TrkA AKFg (8, 30) were generously provided by R. Stephens (NCI-Frederick Cancer Research and Development Center, National Institutes of Health). To generate replication-deficient retroviruses, these vectors were individually transduced into the Binc packaging cell line by the calcium phosphate technique as described previously (31). Two days later, virus-containing supernatant from the pellets was centrifuged at 4 °C for 15 min at 13,000 g and the cleared lystate was then incubated with 0.2 mg/ml polyelectrolyte K at 55 °C for at least 2 h, followed by two sequential extractions with equal volumes of phenol/chloroform (pH 8.0). Following overnight ethanol precipitation and centrifugation, the pellets were dissolved in water containing 5 μg/ml DNase-free RNase at 37 °C for 1 h. Fragmented DNA was resolved by electrophoresis in a 6% Tris borate gel and stained with ethidium bromide. Cell viability was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium (MTS) assay with the electrocoupling reagent phenazine methosulfate according to the manufacturer’s protocol (CellTiter 96 AQ, Promega).

RESULTS

NGF-induced Apoptosis in Medulloblastoma

NGF-induced Apoptosis in Medulloblastoma Cells in Culture—We initially examined the pattern of tyrosine phosphorylation in whole-cell lysates from MED283-TrkA cells and PC12–615 cells, because distinct proteins become tyrosine-phosphorylated following NGF stimulation in PC12 cells (28). The PC12–615 cell line is derived from PC12 cells stably transfected to overexpress TrkA at a significantly higher level than both the parent PC12 cell line and MED283-TrkA cell line (24). We used this cell line as a basis for comparison in order to rule out any difference in protein tyrosine phosphorylation that may be attributed simply to TrkA overexpression. To determine the pattern of NGF-induced protein tyrosine phosphorylation in MED283-TrkA cells, we performed Western blots of total lysates prepared from MED283-TrkA cells treated with NGF for various lengths of time using the anti-phosphotyrosine monoclonal antibody 4G10. We found that tyrosine phosphorylation in MED283-TrkA cells peaked rapidly at 5 min following NGF treatment and then rapidly declined to basal level 30 min after exposure (Fig. 1). Unlike PC12–615 cells, the overall level of tyrosine phosphorylation of which peaked at 1 h post-NGF stimulation, the MED283-TrkA cells underwent a second wave of tyrosine phosphorylation.
phosphorylation that peaked at least 24 h after NGF treatment. Because NGF-induced apoptosis and tyrosine phosphorylation can be blocked by K-252a, a potent inhibitor of Trk tyrosine kinases (24, 33), these findings suggest that NGF activates an apoptotic signaling pathway in the MED283-TrkA cells through a TrkA tyrosine kinase-dependent mechanism.

NGF Induces Apoptosis in MED283 Cells Expressing TrkA Y490F and TrkA Y785F but Not TrkA YY490/785FF—Previous reports demonstrate that signaling pathways induced by activation of the TrkA receptor are mediated in part by mechanisms involving the binding of substrate proteins to tyrosine-phosphorylated sites (7, 8). This prompted us to identify phosphorylation sites on TrkA that may play a critical role in NGF-induced apoptosis. To do so, we engineered MED283 cells to express wild-type TrkA or TrkA receptor mutants with deficiencies in specific signaling capabilities. Retroviral transduction of MED283 cells generated stable cell lines that expressed comparable levels of wild-type and mutant TrkA proteins (Fig. 2A). In vitro ERK kinase assays demonstrated that only mutations involving both tyrosines 490 and 785 (Y490F/Y785F) reduced NGF-mediated ERK activation (Fig. 2B). Because the double mutant did not completely abolish ERK activation, other binding sites such as KFG may also be involved in redundant ERK signaling. In contrast, mutation at tyrosine 490 (Tyr-490) or at tyrosine 785 (Tyr-785) had negligible effects on ERK activation. These results are similar to data from Stephens et al. (8) in PC12 cells. Thus, the putative SHC and PLCγ binding sites, tyrosine 490 and tyrosine 785, respectively, both contribute to ERK activation in MED283 cells, as well as other sites, such as KFG, because the double mutant still retained partial activity (8, 17, 30). Consistent with previous observations that SHC is also critical to phosphotidylinositol-3-OH kinase activation following NGF stimulation (11), the activity of the phosphotidylinositol 3-OH kinase pathway as measured by Akt kinase assays required phosphorylation of tyrosine 490 (Fig. 2C). In contrast, tyrosine phosphorylation of immunoprecipitated PLCγ was not affected by mutation at tyrosine 490 but was abolished by mutation at tyrosine 785 (Fig. 2D). When these transfected cells were then treated with NGF for 72 h and examined for DNA fragmentation, we observed that cells expressing wild-type TrkA receptors as well as the TrkA mutants with a Y490F or a Y785F substitution underwent massive apoptosis (Fig. 2E). However, cells expressing the kinase inactive TrkA mutant (L538N) and the double TrkA mutant (Y490F/Y785F) receptors failed to undergo apoptosis and failed to exhibit the delayed second phase of tyrosine phosphorylation (data not shown).

The similarity between the pathways described above for NGF-induced apoptosis in MB cells and those leading to NGF-induced PC12 cell differentiation (8) led us to hypothesize that NGF may simultaneously activate conflicting growth and differentiation signaling mechanisms in MB cells with the end result being apoptosis. If this mechanism is correct, then TrkA mutations that render this receptor unable to signal cell cycle arrest in PC12 cells should also be unable to induce apoptosis. Because the mutant TrkA with a KFG sequence deletion is

![Fig. 1. Time course of protein tyrosine phosphorylation in MED283-TrkA and PC12-615 cells following NGF stimulation. Shown is the Western blot of total lysate from cells treated with NGF for the indicated periods and probed with the antiphosphotyrosine antibody 4G10.](http://www.jbc.org/lookup/fig/1)

![Fig. 2. A, TrkA protein expression in MED283 cells stably transfected with mutant and wild-type (WT) TrkAs. Western blots were performed on 100 μg/lane total protein lysate from each transfectant pool using a monoclonal antibody to Trk or α-tubulin. B, NGF-stimulated ERK activity in TrkA transfected MED283 cells. Cells were stimulated with NGF for 5 min prior to lysis. An in vitro kinase assay was performed on immunoprecipitated ERK in the presence of [γ-32P]ATP using myelin basic protein (MBP) as substrate. C, NGF-stimulated Akt activity in TrkA transfected MED283 cells. Cells were stimulated with NGF for 5 min prior to lysis. An in vitro kinase assay was performed on immunoprecipitated Akt using glycogen synthase kinase-3 (GSK-3) fusion protein as substrate, and Akt-mediated phosphorylation of GSK-3 was detected with a phospho-specific anti-glycogen synthase kinase-3 polyclonal antibody. D, NGF-stimulated tyrosine phosphorylation of PLCγ in TrkA transfected MED283 cells. Cells were stimulated with NGF for 5 min prior to lysis. PLCγ was immunoprecipitated from total lysates and probed with an anti-phosphotyrosine antibody. E, NGF-induced DNA fragmentation in transfected MED283 cells. Cells were treated with NGF for 72 h and assayed for DNA fragmentation on 2% agarose gel.](http://www.jbc.org/lookup/fig/2)
unable to initiate cell cycle arrest in PC12 cells (30), we examined whether or not MED283 cells expressing this mutant receptor would be protected from NGF-induced apoptosis. Cells expressing the ΔKFG mutant underwent apoptosis comparable to wild-type TrkA (Fig. 3), suggesting that cell cycle arrest may not be critical for the induction of apoptosis by NGF in these MB cells. In summary, the results from these experiments demonstrate an essential role for the phosphorylation of tyrosine residues at position 490 and 785 on TrkA in mechanisms of NGF-induced apoptosis. In addition, they suggest that binding of either SHC or PLC-γ to TrkA may be necessary for downstream activation of apoptotic machinery in MB cells.

**NGF-induced Apoptosis Is Ras-dependent**—Because both tyrosines 490 and 785 mediate signaling through the Ras-ERK pathway, we examined the role of Ras-ERK signaling in NGF-induced apoptosis. Because we could not generate stable clones of MED283-TrkA cells that expressed dominant negative N17Ras (34) by retroviral transfection, we sought to transiently express N17Ras in MED283-TrkA cells using adenoviruses. Cells infected with adenoviruses at a multiplicity of infection of 10 resulted in the expression of N17Ras or GFP at nearly a 100% infection rate as monitored by immunocytochemistry (data not shown). Overexpression of N17Ras completely eliminated ERK phosphorylation at 5 min and 24 h following NGF treatment (Fig. 4A). In contrast, cells infected with adenoviruses expressing GFP alone showed robust ERK phosphorylation at 5 min following NGF treatment. Significantly, cells infected with N17Ras were partially protected from NGF-induced apoptosis, as evidenced by a decrease in the extent of DNA fragmentation and an increase in cell viability (Fig. 4, B and C). Taken together, these data indicate that suppression of endogenous Ras activity diminishes the extent of apoptosis induced by treating MB cells with NGF. Thus, Ras plays a critical role in the apoptotic pathways that are induced by NGF-mediated activation of TrkA receptors in MB cells.

**NGF-induced Apoptosis Does Not Require MEK/ERK**—To investigate whether NGF-induced apoptosis was influenced by signaling through the major MEK/ERK pathway (10, 35), we monitored cell viability and apoptosis in MED283-TrkA cells pretreated with the specific inhibitors of MEK PD98059 (50 μM) or U0126 (10 μM) (36, 37). Immunoprecipitated ERK from untreated and treated cells was used to phosphorylate myelin basic protein in the presence of [γ-32P]ATP to confirm the inhibition of MEK by PD98049 (Fig. 5A) and U0126 (data not shown) in MED283-TrkA cells. NGF-induced ERK phosphorylation was inhibited by pretreating cells with PD98059 or U0126. However, neither PD98059 (Fig. 5, B and C) nor U0126 (data not shown) affected cell viability and apoptosis, as shown by MTS and DNA fragmentation assays. Similar results were obtained when medium containing PD98059 or U0126 was changed every 12 h (data not shown). Thus, we conclude from these studies that the induction of apoptosis by NGF in MED283-TrkA cells is mechanistically linked to Ras activation but not to the major MEK/ERK-dependent signaling pathway.

**NGF-induced Apoptosis Requires Caspase Activity**—Because caspases, a family of cysteine proteases that specifically cleave proteins after Asp residues, have been implicated in signaling pathways that regulate the initiation of apoptotic cell death mechanisms (38), we investigated whether or not caspase activation was involved in NGF-induced apoptosis in MED283-TrkA cells. To do this, we examined the effects of BAF, a general caspase inhibitor (39), on the extent of DNA fragmentation associated with apoptosis of the MED283-TrkA cells following NGF treatment. As shown in Fig. 6, BAF (20 μM) completely inhibited DNA fragmentation in NGF-treated samples, and it also inhibited background DNA fragmentation in untreated controls. These results demonstrate an essential role...
of caspases in DNA fragmentation in NGF-induced apoptosis as well as background apoptosis in MB cells.

DISCUSSION

The data presented here indicate that NGF activates the TrkA receptor and induces apoptosis in MB cells through activation of a novel Ras-dependent pathway (Fig. 7). We showed that the elimination of both of the tyrosine residues that are specific interaction sites for SHC and PLC-γ in TrkA resulted in a functionally defective receptor, thereby linking these tyrosine residues to critical events in the apoptotic signaling pathways that are initiated by NGF treatment. The requirement of Tyr-490 and Tyr-785 does not imply that activation of SHC or PLC-γ alone is sufficient to induce apoptosis because other substrates may also bind to these tyrosines and collaborate to induce apoptosis, such as fibroblast growth factor receptor substrate-2/SNT (8, 17, 30). However, this is not likely given the Ras dependence of TrkA-mediated apoptosis, and mutation of the fibroblast growth factor receptor substrate-2 binding site did not prevent apoptosis.

NGF-induced Apoptosis in Medulloblastoma

The data presented here also indicate that the common Ras effector MEK is not required for NGF-induced apoptosis, indicating that a novel pathway activated through Ras or Raf is important for this process. This result is consistent with a report from Kim et al. (40), who also demonstrated that ERK activation, but not p38 mitogen-activated protein kinase, is also dispensable in NT-3-induced apoptosis in a trkC-expressing MB cell line. Preliminary results in our laboratory suggest that the p38 mitogen-activated protein kinase pathway may play a role in NGF-induced apoptosis as well. These results suggest the likelihood of a common pathway in the mechanism leading to NGF- and NT-3-induced apoptosis in MBs.

Ras signaling is typically associated with cell proliferation, differentiation, and survival. However, activated Ras may also increase cellular sensitivity to signals that induce apoptosis. For example, mice deficient in p120 GTPase-activating protein, a negative regulator of Ras, display a striking increase in the apoptotic death of cells in the anterior neural tube and cranial neural crest (41). Conceivably, medulloblastomas may recapitulate some properties of these cells and thereby undergo apoptosis upon Ras activation. In fibroblasts immortalized with the activated c-myc oncogene, oncogenic Ras is an effective promoter of apoptosis through Raf (42). The results presented here share many features with oncogenic Ras-induced apoptosis. First, oncogenic-Ras initiated apoptosis in fibroblasts that overexpress c-myc is p53-independent (43). NGF-induced apoptosis is also p53-independent because the Daoy MB cell line carries an inactivating p53 mutation but nevertheless undergoes apoptosis in response to NGF (24). Second, inactivation of NF-κB induction by cycloheximide exacerbates apoptosis in both systems (43). Third, c-Myc cooperates with Ras to induce apoptosis in transformed fibroblasts, and MB cell lines in vitro tend to express high levels of c-myc or demonstrate c-myc amplification, including the MED283 cell line (44, 45). And fourth, PD98059 fails to prevent apoptosis in both systems (42). Because oncogenic Ras-mediated apoptosis is also Raf-dependent and MEK-independent (42), it would be important to determine the role of Raf in NGF-induced apoptosis as well.
In summary, this work demonstrates a novel role for Ras that is independent of MEK/ERK in NGF-induced apoptosis and highlights a potential strategy of modulating this pathway for treatment of MBs. We suggest that this apoptotic pathway could play a role in the initiation and/or progression of MBs, as well as other neural tumors, and that it also may be one of several neurotrophin responses that regulate the normal development, maturation, or senescence of the nervous system.

Acknowledgments—We thank M. V. Chao (Cornell University Medical College) for the PC12–615 cell line, G. M. Cooper (Harvard Medical School) for the N17Ras vector, D. R. Kaplan (Montreal Neurological Institute) and R. M. Stephens (NCI-Frederick Cancer Research and Development Center) for the pLNCX-TrkA vector, Farhad Abtahian for technical assistance, and Dan Skovrisky for critical review of the manuscript.

REFERENCES

A Novel Apoptotic Pathway Induced by Nerve Growth Factor-mediated TrkA Activation in Medulloblastoma
Thomas T. Chou, John Q. Trojanowski and Virginia M.-Y. Lee

doi: 10.1074/jbc.275.1.565

Access the most updated version of this article at http://www.jbc.org/content/275/1/565

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 22 of which can be accessed free at http://www.jbc.org/content/275/1/565.full.html#ref-list-1