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Resistance to Chemotherapy Mediated by TrkB in Neuroblastomas[1]

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Abstract

Neuroblastoma is a common childhood tumor derived from the peripheral nervous system. Favorable neuroblastomas usually express TrkA, the receptor for nerve growth factor (NGF), whereas unfavorable, *MYCN*-amplified neuroblastomas usually express TrkB and its ligand, brain-derived neurotrophic factor (BDNF). Here, we provide evidence that the TrkB-BDNF pathway is associated with enhanced survival and resistance to chemotherapy in neuroblastoma. We transfected the neuroblastoma line SH-SY5Y, which has endogenous expression of BDNF, with a full-length TrkB expression vector, and obtained clones with moderate or high levels of expression. Cells were exposed *in vitro* to chemotherapy agents used to treat neuroblastomas: doxorubicin, etoposide (VP16), and cisplatin. Chemoresistance was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell survival and by ELISA for cell death. In all cases, the TrkB-expressing subclones were more resistant to treatment than the parent line. Furthermore, when the TrkB tyrosine kinase was blocked with the Trk-specific inhibitor CEP-2563, or by neutralizing antibody to BDNF, sensitivity to chemotherapy was significantly increased. We also found constitutive phosphorylation of AKT at the Ser-473 site in TrkB transfectants, whereas there was only a minimal level of constitutive phosphorylation of AKT in SY5Y cells. These results show that the TrkB-BDNF pathway provides a survival advantage when exposed to DNA-damaging reagents, and, therefore, this autocrine pathway may play an important role in mediating the drug-resistant phenotype associated with TrkB-expressing neuroblastomas. Activation of PI3K/AKT survival pathway may contribute to the increased drug resistance in TrkB-expressing neuroblastomas.

Introduction

Neuroblastoma is the most common extracranial solid tumor in children. It is derived from the neural crest and usually arises in the adrenal medulla or along the sympathetic chain (1). Biologically and clinically, neuroblastomas can be divided into at least two groups: one with favorable biological features (favorable histology, hyperdiploidy, TrkA expression) and another with unfavorable features (unfavorable histology, near-diploidy, *MYCN* amplification, 1p deletion, TrkB expression). Favorable neuroblastomas are prone to undergo either differentiation or apoptosis, whereas unfavorable neuroblastomas are frequently metastatic and have poor prognosis. Although tumors with unfavorable biological features may respond to chemotherapy initially, they usually recur and are then resistant to further treatment. Indeed, the survival of the patients who have unfavorable neuroblastomas is under 40%.

Neurotrophins are important for the growth, survival, and differentiation of normal sympathetic neurons. The biologically active receptors for the neurotrophins nerve growth factor (NGF), BDNF[3] and neurotrophin-3 are TrkA, TrkB, and TrkC, respectively. Neurotrophin-3 can bind to all of the Trk receptors, and neurotrophin-4 binds preferentially to TrkB. Most neuroblastomas also express at least one member of the Trk tyrosine kinase receptor family. TrkA is expressed mainly in favorable neuroblastomas, whereas TrkB and its ligand BDNF usually are expressed in unfavorable neuroblastomas (2–7). TrkC is expressed in a subset of the TrkA-expressing neuroblastomas, but its biological and clinical significance is less clear (8–10). The TrkB-BDNF pathway appears to be an autocrine pathway that is important for the survival of biologically unfavorable neuroblastoma cells (4, 11, 12).

Previous reports have shown that BDNF increases chemoprotection for neuroblastoma cells induced to express TrkB by retinoic acid treatment (13). Furthermore, BDNF has been reported to protect neuroblastoma cells from vinblastine cytotoxicity (12). Here, we examined the potential contribution of the TrkB/BDNF autocrine pathway to the chemoresistance of TrkB-expressing neuroblastomas. We stably transfected full-length TrkB into the SH-SY5Y cell line, which has no detectable levels of TrkA, TrkB, or TrkC expression, and studied the impact of exogenous TrkB on the cells exposed to chemotherapy *in vitro*. We demonstrate that the TrkB/BDNF autocrine survival pathway plays an important role in mediating resistance to a variety of chemotherapy agents.

Materials and Methods

Cell Cultures. The SH-SY5Y (SY5Y) human neuroblastoma cells were maintained in an atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% FBS, 1% oxaloacete-pyruvate-insulin (OPI) supplement, 1% glutamate, and 50 μg/ml gentamicin.

Transfection of TrkB. The full-length TrkB cDNA was cloned into the pcDNA3 plasmid vector and transfected into SY5Y cells by electroporation. Stable transfected cells were selected in 400 μg/ml Geneticin. The Geneticin-resistant cells were further subcloned to single-cell clones demonstrating moderate (G12) or high (G7) levels of TrkB expression. The expression of TrkB protein for the single-cell clones was characterized by Western blotting.

Semiquantitatve RT-PCR of BDNF. Total RNA was extracted from the SY5Y parental cells and TrkB expressing subclones and semiquantitative RT-PCR was carried out as described previously (14). Primer sequences for human BDNF were as follows: sense, 5′-CTGCAAACATGTCCATGAGG-3′; antisense, 5′-CCTGCAGCCTTC TTTTGTGT-3′. The primers were biotinylated at their 5′ ends.

Immunoblotting and Immunoprecipitation. For the analysis of TrkB expression, transfected cells were grown to confluency in T-75 flasks and then collected. The cell pellets were lysed in 100–200 μl of NP40 lysis buffer [1% NP40, 20 mM Tris (pH 8.0), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, 20 μM leupeptin, and 1 mM sodium vanadate] on ice for 20 min. Cell lysates were cleared by centrifugation for 20 min at 14,000 $\times$ *g*, and protein content was quantitated

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[3] The abbreviations used are: BDNF, brain-derived neurotrophic factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; MAPK, mitogen-activated protein kinase; VP, etoposide; DOX, doxorubicin; CP, cisplatin; PI3K, phosphatidylinositol 3′-kinase.

by Bio-Rad protein assay. TrkB expression was analyzed by Western Blot using anti-pan Trk antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and detected by Enhanced Chemiluminescence (ECL) Western Blotting System (Amersham Corp., Arlington Heights, IL).

For an analysis of receptor autophosphorylation, AKT and MAPK expression, the cells were grown in 10-cm^2 dishes to 80% confluence in RPMI 1640 supplemented with 2% FBS overnight. For studying blockage effect of neutralizing antibody or tyrosine kinase inhibitors, cells were incubated with anti-BDNF antibody (20-μg/ml final concentration; Oncogene Research, San Diego, CA) or CEP-2563 (100-nM final concentration; Cephalon, Inc., West Chester, PA) in low-serum medium for 48 h or 24 h, respectively. Cells were rapidly lysed in lysis buffer as described above with the addition of 1 M NaF and 500 mM sodium PP_I, maintained on ice, and processed as described previously. One mg of total protein was immunoprecipitated with anti-Trk antibody and detected by Western Blotting with the anti-phosphotyrosine antibody (Santa Cruz Biotechnology). Total protein (250 μg) was analyzed by Western Blot using an anti-Phospho-AKT473 (Cell Signaling, Beverly, MA) or an anti-phospho-MAPK (Promega, Madison, WI) antibody. Reprobing with anti-AKT (Cell Signaling) and anti-MAPK (Santa Cruz) antibody was performed to ensure equal loading of protein.

Cytotoxicity Assay. SY5Y (TrkB-null), G12 (TrkB-moderate), and G7 (TrkB-high) cells were seeded into 96-well plates at a density of 3×10^4 cells/well in RPMI medium with 2% FBS. All of the experiments were performed in triplicate and repeated at least three different times. The cells were treated with VP (5 μg/ml; Sigma), CP (5 μg/ml); Sigma), and DOX (0.5 μg/ml; Sigma) for 24 h starting on the 2nd day after replating. For combination treatments, anti-BDNF antibody (20 μg/ml) or CEP-2563 (100 nM) was added at the same time in addition to the drugs. A colorimetric MTT assay was then performed to measure cell survival (15). A multiwell scanner was used to measure the absorbance at 570–630 nm dual wavelengths. The untreated controls were assigned a value of 100%.

Cell Death ELISA. SY5Y, G12, and G7 cells were seeded into 96-well plates at a density of 3×10^3 cells/well in 2% FBS RPMI medium. Experiments were performed in triplicate. Cells were treated as detailed in the cytotoxicity assay. Apoptosis was measured by the detection of fragmented DNA from the nucleus to cytoplasm using a colorimetric assay, Cell Death Detection ELISA kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocols. Untreated cells were assigned as controls. The extent of apoptosis of each cell line under each condition was represented by the apoptosis rate (the absorbance of cells treated with the cytotoxic agent divided by the absorbance of the untreated cells).

Statistical Analysis. The results of the MTT assay and cell death ELISA were analyzed by an unpaired Student *t* test.

Results

TrkB and BDNF Expression in TrkB Transfectants. Previously, reports have shown that BDNF increases chemoprotection for neuroblastoma cells that have been induced to express TrkB by retinoic acid treatment (13). Retinoic acid has many effects on the proliferation and differentiation of neuroblastoma. To study the long-term effect of TrkB expression in neuroblastoma, we stably transfected full-length TrkB into SY5Y cells. Stable transfected cells were selected by Geneticin and further subcloned to single-cell clones. We used representative clones G12 and G7 in our studies. As demonstrated by Western blot, TrkB protein was undetectable in parental SY5Y cells. The TrkB-transfected single-cell clones G12 and G7 have intermediate- or high-level expression of TrkB protein, respectively (Fig. 1*A*). Semiquantitative RT-PCR detected endogenous expression of BDNF mRNA in parental SY5Y cells and in the TrkB transfectants (G12 and G7). **(**Fig. 1*B***)**. The level of BDNF mRNA in the transfectants is somewhat higher than in the parental SY5Y cells. The coexpression of TrkB and endogenous BDNF appears to represent an autocrine loop in these cells.

Anti-BDNF Neutralizing Antibody and CEP-2563 Block the TrkB-BDNF Autocrine Loop in TrkB Transfectants. We investigated whether endogenous BDNF could activate the TrkB receptor and initiate signal transduction in TrkB-transfected neuroblastoma cells. We also examined the ability of a neutralizing antibody to BDNF, or a Trk-selective inhibitor—CEP-2563, to interfere with the autocrine activation loop. G7 cells were grown under low-serum conditions for 12 h. Autocrine activation of TrkB by endogenous BDNF was examined in untreated cells. To evaluate the effect of inhibiting this pathway, G7 cells were treated with anti-BDNF neutralizing antibody for 48 h or with the Trk-selective tyrosine kinase inhibitor CEP-2563 for 24 h. Autophosphorylation of $p145^{TrkB}$ in Trk expressing G7 cells was examined by immunoprecipitation with anti-Trk antibody followed by Western blot with anti-phosphotyrosine antibody. In the absence of treatment, autophosphorylation of $p145^{TrkB}$ was detected, suggesting that the autocrine loop is constitutively active. However, both anti-BDNF neutralizing antibody and CEP-2563 block autophosphorylation of $p145^{TrkB}$ almost completely (Fig. 1, *C* and *D*).

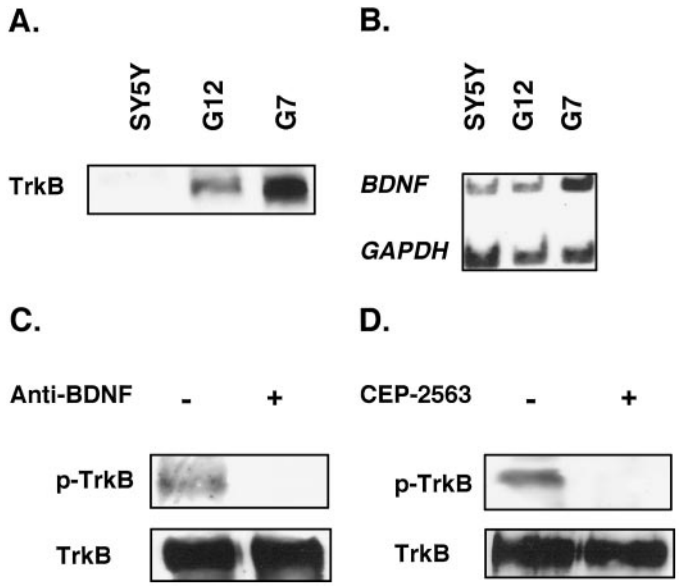

Fig. 1. Expression of TrkB protein and BDNF mRNA in SY5Y cells and its transfectants, and tyrosine phosphorylation of $p145^{TRK\text{-}B}$ in the TrkB-transfected clone G7. *A*, TrkB protein expression in SY5Y, G12, and G7 cells detected by immunoblotting. *B*, BDNF mRNA expression in SY5Y, G12, and G7 cells detected by semiquantitative RT-PCR compared with GAPDH expression as a control. In *C* and *D*, cells were cultured in 2% FBS overnight, and then treated with either 20 μg/ml BDNF (*C*) or 100 nM CEP-2563 (*D*) for 48 h or 24 h, respectively. The cell lysates were immunoprecipitated with an anti-Trk antibody and blotted with an anti-phosphotyrosine antibody.

Exogenous Expression of TrkB Enhances Survival and Reduces Apoptosis of SY5Y Cells Treated with Chemotherapeutic Agents. We analyzed the effects of TrkB expression on the response of neuroblastoma cells to chemotherapy *in vitro*. Three chemotherapeutic agents used commonly in the treatment of neuroblastoma—DOX, VP, and CP—were used. The survival percentage of the TrkB-transfected cells, as determined by MTT assay, was significantly higher than the SY5Y parental cells after treatment with all three of the drugs **(**Fig. 2*A***)**. Indeed, the higher the level of TrkB expression, the higher the percentage of survival after treatment with chemotherapeutic agents. Conversely, apoptosis rates of TrkB transfectants were significantly lower than the SY5Y parental cells after treatment with the three drugs above (Fig. 2*B*). The higher the expression level of TrkB, the lower the rate of apoptosis in response to chemotherapy.

CEP-2563 and anti-BDNF Neutralizing Antibodies Increase the Sensitivity of TrkB Transfectants to Chemotherapeutic Agents. When TrkB-expressing neuroblastoma cell lines were treated with both anti-BDNF neutralizing antibody and a chemotherapeutic agent, the percentage of surviving cells was significantly decreased when compared with treatment with a chemotherapeutic agent

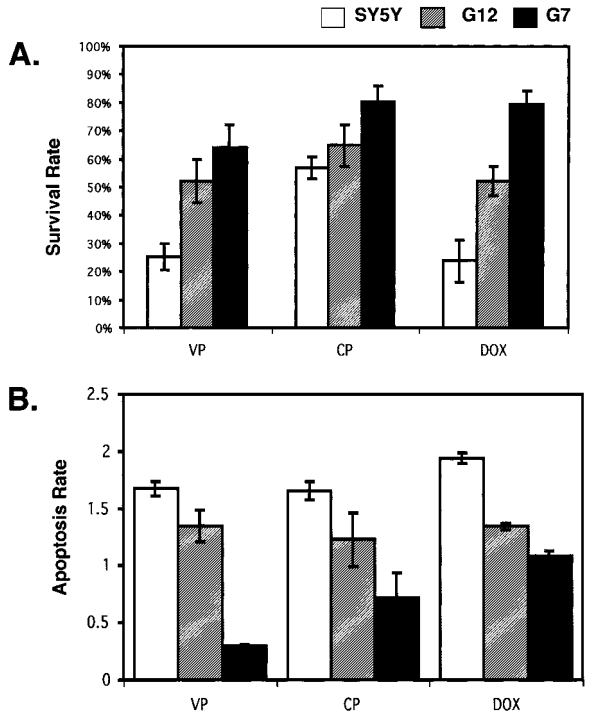

Fig. 2. TrkB expression increases chemoresistance in neuroblastoma cells. In *A*, SY5Y cells and TrkB transfectants were cultured in medium containing 2% FBS in 96-well plates and treated with VP (2.5 μg/ml), CP (5 μg/ml), or DOX (0.5 μg/ml) for 24 h. Cell survival rates were determined by MTT assay. Data points are the mean of triplicate samples (*error bars*, $\pm$ SD) and represent the percentage of cells surviving compared with untreated cells. Representative results shown are from one of at least three separate experiments. The differences in survival rates between SY5Y and either G12 or G7 cells are significant ($P < 0.01$ for all cases except for G12 cells treated with CP, which is $P = 0.04$). In *B*, cells were treated as described in *A*. The apoptosis rate was determined by DNA fragmentation cell death ELISA. *Data points*, the mean of triplicate samples (*error bars*, $\pm$SD). The differences in apoptosis rates between SY5Y and G7 cells are significant ($P < 0.01$ for VP16 and DOX and $P = 0.06$ for CP). The difference in apoptosis rate between SY5Y and G12 did not reach significance for cells treated with VP16 and CP, but the difference for cells treated with DOX is significantly different ($P = 0.01$).

alone (Fig. 3*A*). The results were similar for all three drugs tested (VP, CP, DOX). When cells were treated with both CEP-2563 and chemotherapy, the percentage survival for the TrkB transfectants was also significantly decreased compared with treatment with chemotherapy alone (Fig. 3*B*). In contrast, the addition of either the anti-BDNF neutralizing antibody or 100 nM CEP-2563 to chemotherapy did not alter the survival of SY5Y cells as compared with chemotherapy alone.

PI3K Pathway Is Activated by Autocrine TrkB-BDNF Pathway in TrkB Transfectants. BDNF can activate several signal transduction systems including the PI3K and MAPK pathways. To determine whether the PI3K pathway is responsible for increased chemoresistance in TrkB transfected cells, we assayed PI3K activity by Western blot using a phospho-AKT antibody that specifically recognizes activated AKT. Reprobing with an anti-AKT antibody was used to ensure an equal amount of protein loading in each lane. Phosphorylated AKT473 was detected in G12 and G7 cells and was not detected in parent SY5Y cells under basal conditions (Fig. 4*A*), indicating activation of AKT by an autocrine TrkB-BDNF pathway in the transfectants. In addition, we found the activated AKT can be blocked by the PI3K-specific inhibitor LY294002. When we treated cells with LY294002 in addition to chemotherapeutic agents, we found more restored chemosensitivity in G7 cells than in parental SY5Y cells (data not shown). We also determined the contribution of MAPK pathway to survival by examining the expression phospho-MAPK by Western blot. Basal expression of phospho-MAPK was found both in SY5Y and in TrkB transfected cells, and the expression level of phospho-MAPK did not increase with TrkB expression (Fig. 4*B*).

Discussion

The expression of TrkB and BDNF in neuroblastomas is primarily found in *MYCN*-amplified tumors and associated with a poor outcome (4). Here we addressed the effect of TrkB expression on the resistance of neuroblastoma cells to conventional chemotherapeutic agents. The SY5Y cell line has endogenous BDNF expression but undetectable levels of endogenous TrkB expression. We demonstrate that the TrkB-BDNF autocrine pathway is active in SY5Y-TrkB clones after transfection. Steady-state autophosphorylation of TrkB by endogenous BDNF was observed in the TrkB transfectants. This autophosphorylation can be blocked by anti-BDNF antibody or by the Trk-specific tyrosine kinase inhibitor CEP-2563. Furthermore, increased resistance to chemotherapy agents was observed in the TrkB transfectants compared with the parental SY5Y cells. Blocking the TrkB-BDNF pathway by anti-BDNF-neutralizing antibody or by the Trk tyrosine kinase inhibitor CEP-2563 sensitizes the cell response to chemotherapy. When combined with chemotherapy, anti-BDNF antibody decreases the cell survival toward baseline by an average of 35%, and CEP-2563 decreases it by an average of 55%, compared with the chemotherapy alone. Thus, the activated autocrine TrkB-BDNF pathway probably plays an important role in increased chemoresistance observed clinically in TrkB-expressing tumors.

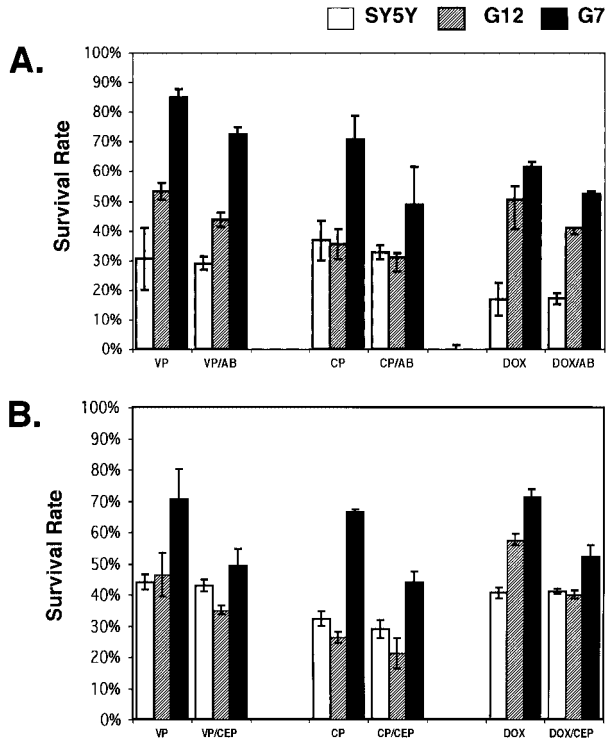

Fig. 3. CEP-2563 (CEP) or anti-BDNF (AB) antibody sensitizes TrkB transfectants to chemotherapy. SY5Y cells and TrkB transfectants were cultured in medium containing 2% serum in 96-well plates and treated with VP (2.5 μg/ml), CP (5 μg/ml), or DOX (0.5 μg/ml) together with either anti-BDNF antibody (20 μg/ml) or CEP-2563 (100 nM) for 24 h. Cell survival rate was determined by MTT assay. *Data points*, the percentage of cells surviving compared with untreated cells [the mean of triplicate samples (*error bars*, $\pm$ SD)]. Representative results shown are from one of at least three separate experiments. *A*, cells treated with anti-BDNF antibody. The difference in survival rates between G7 cells treated with chemotherapy plus anti-BDNF antibody compared with chemotherapy alone is significant ($P < 0.01$ for cells treated with DOX) and marginal ($P < 0.1$ for cells treated with VP16 and CP). *B*, cells treated with the Trk inhibitor CEP-2563. The difference in survival rates between G7 cells treated with chemotherapy plus CEP-2563 compared with chemotherapy alone is significant ($P < 0.01$ for all).

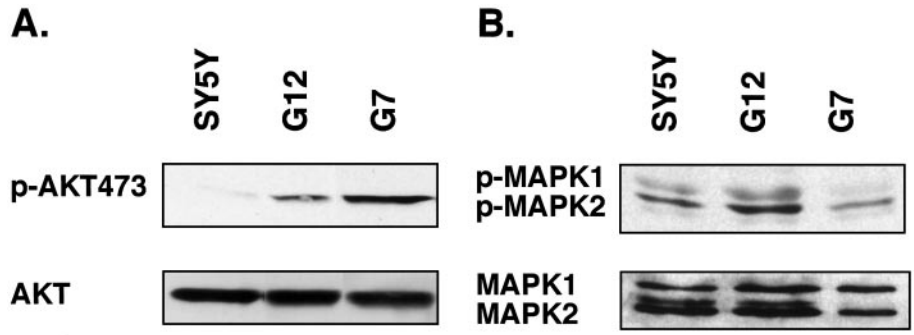

Fig. 4. Expression of AKT and MAPK protein in SY5Y cells and its transfectants. *A*, phospho-AKT473 and AKT protein expression detected by Western blot. *B*, phospho-MAPK and MAPK protein expression detected by Western blot.

The TrkB-BDNF pathway promotes cell survival and protects neuronal cells from injury. There is a report that BDNF promotes survival and differentiation of striatal projection neurons (16). Treatment with BDNF-antisense oligonucleotides reduced neuronal survival by 35%, and the addition of BDNF or neurotrophin-3 rescues cells from death. The BDNF autocrine loop also prevents cell death in adult sensory neurons (11). Overexpression of BDNF enhances sensory enervation and selectively increases neuron numbers (17). In cortical neurons, BDNF promotes protection from camptothecin-induced apoptosis (18).

The TrkB-BDNF pathway promotes cell survival and protects cells from DNA damaging agents in neuroblastoma cells (13, 19). Induction of TrkB expression in the SY5Y and NGP neuroblastoma cell lines promoted dependence on BDNF for survival. An anti-BDNF neutralizing antibody induces dose-dependent apoptotic death in these cells. The neutralizing antibody also induces apoptotic death in TrkB-expressing cell line SMS-KCN (20). BDNF increases the proliferation of TrkB-expressing SY5Y cells, whereas it has no effect on the cells without inducing TrkB expression by retinoic acid (12). BDNF also promoted survival of the *MYCN*-amplified neuroblastoma cell line NB1643 during treatment with CP (13). Taken together, the TrkB-BDNF pathway plays an important role in survival and protection from injury in both normal neurons and neuroblastoma cells. Thus, this pathway may be responsible for increased survival of TrkB-expressing neuroblastoma cells when exposed to chemotherapy.

Full-length TrkB is expressed almost exclusively in biologically unfavorable neuroblastomas. Nakagawara *et al.* (4) examined a series of 74 primary tumors and found 68% of neuroblastomas express BDNF, 36% express TrkB, and 31% express both in primary neuroblastomas. Others have confirmed that human neuroblastomas with unfavorable biological features express high levels of BDNF mRNA and its variants (21). Therefore, an autocrine or paracrine TrkB/BDNF pathway may contribute to the unfavorable outcome in primary neuroblastomas.

BDNF binding to TrkB leads to the activation of at least three major signaling pathways (Ras/MAPK, PI3K, and PLC-Y1; Ref. 4). MAPK and PI3K pathways are thought to be involved in BDNF-induced survival. Some reports have shown that both MAPK and PI3K are required to promote the survival of sympathetic or cerebellar neurons by BDNF (22, 23). One group showed that the MAPK pathway plays a major role in BDNF neuroprotection against camptothecin in central nervous system neurons, whereas the PI3K pathway is the dominant survival mechanism for BDNF protection against serum withdrawal (18). Other groups have reported that the PI3K pathway is necessary for survival mediated by BDNF in motoneurons and in SY5Y cells treated with retinoic acid, whereas the MAPK pathway is not necessary (24, 25). Here we have shown autocrine activation of AKT by the TrkB-BDNF pathway in TrkB transfectants. This activation can be blocked by the PI3K-specific inhibitor LY294002, leading to increased chemosensitivity in TrkB transfectants. We also found basal activation of MAPK in both SY5Y cells and TrkB-transfected cells, and the level of activated MAPK did not correlate with the expression level of TrkB. Taken together, we think PI3K pathway is primarily responsible for TrkB-mediated chemoresistance in our system.

Blocking the TrkB-BDNF pathway may be an important approach to overcome chemoresistance associated with biologically unfavorable neuroblastomas. We have shown previously that the Trk-specific inhibitor CEP-751 (a metabolite of CEP-2563) alone has an antitumor effect on neuroblastoma mouse xenografts (26). We also demonstrated that this inhibitor has a more potent antitumor effect in TrkB-expressing mouse xenografts (27). We tested the Trk-specific inhibitor CEP-2563 *in vitro* and found that it, indeed, partially reversed the chemoresistance of TrkB transfectants *in vitro*. Hence, using CEP-2563 may be a promising method to overcome chemoresistance in unfavorable neuroblastomas. We are currently testing the effect of combination treatment of CEP-2563 and chemotherapy in mouse xenografts.

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