Cisplatin-induced cytotoxicity is blocked by brain-derived neurotrophic factor activation of TrkB signal transduction path in neuroblastoma

Jerry Jaboin[a,1], Audrey Hong[a], Chong Jai Kim[b], Carol J. Thiele[a,*]

[a]*Cell and Molecular Biology Section, Pediatric Oncology Branch, Center for Cancer Research, NCI, 10 Center Dr. MSC-1928, Bethesda, MD, USA*
[b]*Department of Pathology, Seoul National University College of Medicine, Seoul, South Korea*

Abstract

We evaluated the ability of brain-derived neurotrophic factor (BDNF) to decrease the chemosensitivity of neuroblastoma cells to cisplatin. Two cell lines, one derived from SH-SY5Y (SY5Y-TB8) and the other from SK-N-AS (AS-TB8), transfected with a TrkB plasmid were generated, and used to assess the effects of activation of the TrkB signal transduction path on cisplatin (Cis) induced apoptosis. BDNF treatment of each of the TrkB expressing cells blocked cisplatin-induced cell death. BDNF's ability to rescue the cells from cisplatin-induced cell death was inhibited by treatment with the Trk tyrosine kinase inhibitor, K252a, and the phosphatidylinositol 3′-kinase (PI)-3-kinase inhibitor, LY294002. This indicates that the activation of the TrkB path through PI-3-kinase is required for BDNF's survival-promoting effects.
© 2003 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Neuroblastoma; cisplatin; TrkB; Brain-derived neurotrophic factor; Phosphatidylinositol 3′-kinase

1. Introduction

Neuroblastoma (NB) is a pediatric solid tumor derived from neural crest precursor cells [1]. It has been shown that favorable-prognosis NB tumors express relatively high levels of TrkA, whereas cell lines and tumors from patients with poor prognosis are associated with expression of brain-derived neurotrophic factor (BDNF) and its receptor, TrkB [1–3]. Additionally, several studies have indicated that BDNF increases NB cell survival [2,4,5], neurite extension [6,7], cell invasion [4] and protects cells from chemotherapy [8–10]. BDNF promotes survival in a variety of normal neuronal cell types including striatal neurons [11], and dorsal root ganglionic neurons [12]. BDNF also protects hippocampal neurons [13] and motor neurons from axotomy-induced cell death [13], hippocampal neurons from nitrous oxide induced apoptosis and cerebellar granule neurons from metabolic and excitotoxic

insults [14–17]. Thus for normal neurons BDNF, usually via activation of TrkB, protects cells from injury.

We hypothesize that activation of TrkB would protect NB cells from chemotherapy. In NB cells expressing a transfected TrkB or endogenous TrkB induced after retinoid treatment, we have demonstrated that BDNF could partially protect from vinblastine-induced cell death [8]. To investigate the signaling system by which BDNF protects NB cells from chemotherapy, we developed TrkB expressing SY5Y cell lines [18] and TrkB-expressing AS cell lines. Recently we found that activation of the TrkB via the propidium iodide (PI)-3 kinase path BDNF/TrkB rescues NB cells from etoposide-induced cytotoxicity [10]. Similarly, in this study we find that BDNF stimulation of TrkB rescues NB cells from apoptosis induced by cisplatin. Additionally, we find that the BDNF-induced rescue of cells from cisplatin can be blocked by inhibiting Trk tyrosine kinase activity, and selectively targeting the PI-3-kinase path.

2. Materials and methods

2.1. Construction of vectors and establishment of stable transfectants

A 3.1 kb fragment of rat TrkB spanning a full coding region was subcloned into the pBSTR1 vector [18] and transfected into the human NB cell lines SH-SY5Y and SK-N-AS using lipofectAMINE (Life Technologies, Inc.). Stable transfectants (SH-SY5Y-TB8 (SY5Y-TB8); SK-N-AS-TB8 (AS-TB8)) were obtained after selection with puromycin (0.5 μg/ml) [18].

2.2. Cell culture

Cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine and antibiotics as previously described [18]. To maintain selection pressure the AS-TB8 and SY5Y-TB8 cells were cultured in puromycin (0.5 μg/ml).

2.3. Reagents

Recombinant human BDNF (100 ng/μl) (Pepro-Tech, Inc.) stocks were prepared as directed and stored at $-20\,°C$. Cisplatin was obtained from Sigma, and 1 mg/ml stocks prepared according to the company's specification, and stored at $-20\,°C$. The pharmacological inhibitors, LY294002 (1 mM) and K252a (1 mM) were obtained from Sigma, and reconstituted according to manufacturer's specification.

2.4. Immunoblotting/immunoprecipitation

For protein analysis, cells were washed in phosphate-buffered saline (PBS), centrifuged and cell pellets kept in $-70\,°C$ until analysis and prepared as described previously [6]. The cells were then lysed in Nonidet P-40 (NP-40) lysis buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 500 μM sodium orthovanadate in ice-cold PBS, and immuno-precipation was performed as previously described [10]. Akt phosphorylation changes were analyzed by Western blotting analysis of 50 μg of protein loaded onto 12% Tris–Glycine bis-acrylamide gels, and probed with rabbit monoclonal antibodies to phosphoAkt (Cell Signaling). Total Akt protein levels were determined by Western blotting analysis of 50 μg of protein with rabbit monoclonal antibodies to Akt. Signals were detected using enhanced chemiluminescence reagents (Pierce).

2.5. Cell survival analysis

NB cells (1×10^4) per well were plated into 96-well plates. After 16–24 h, the cells were treated with control media or BDNF (100 ng/ml) for 15 min. The next day, the indicated concentrations of cisplatin were added for 2 h. The culture supernatant was then removed, and fresh media added for the remainder of the culture period. Cell number was assessed 48 h later using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [10]. Each value represents six replicates, and each experiment was repeated 2–3 times.

3. Results

3.1. Characterization of TrkB-expressing NB cell lines

In order to evaluate the effect of TrkB activation on cisplatin chemosensitivity, we utilized TrkB-transfected cell lines from SK-N-AS (AS-TB8) and SH-SY5Y (SY5Y-TB8) cell lines for evaluation. Upon treatment by BDNF (100 ng/ml) for 5 min, autophosphorylation of TrkB can be detected in each of the cell lines (Fig. 1A). In addition, TrkB phosphorylation is abrogated by pre-treatment for 30 min with the selective Trk kinase inhibitor, K252a (500 nM), indicating intact TrkB signal transduction in these cells (Fig. 1A). Activation of the TrkB path leads to activation of several downstream signaling paths, one of which is the PI-3-kinase pathway. A key target of the PI-3-kinase pathway is phosphorylaion of Akt (PO_4Akt). Following BDNF activation, PO_4Akt is detected in both the AS-TB8 and SY5Y-TB8 cell lines
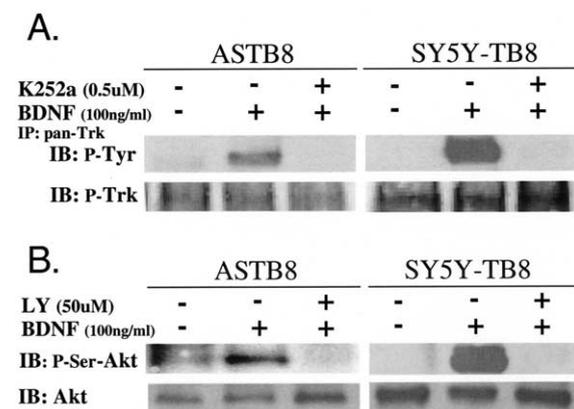

Fig. 1. Functional TrkB Expression AS-TB8 and SY5Y-TB8 cells were cultured for 16–24 h in normal RPMI with 10% fetal bovine serum. (Panel A): TrkB Path; on the 3rd day, the cells were treated with or without K252a (500 nM) for 30 min, stimulated with or without BDNF (100 ng/ml) for 5 min, and subsequently washed and harvested. Protein lysate was extracted from each condition, 500 μg protein was then immunoprecipitated with rabbit polyclonal anti-pan Trk antibody (C-14) and subjected to Western analysis (as described in Section 2) for evaluation of TrkB autophosphorylation and total TrkB levels. (Panel B): PI-3-kinase Path; on the 3rd day, the cells were treated with or without LY294002 (LY) (50 μM) for 30 min, stimulated with or without BDNF (100 ng/ml) for 5 min, and subsequently washed and harvested. Protein lysate was extracted from each condition, and 50 μg protein was analyzed for PO_4-Akt and total Akt expression by Western blot analysis.

(Fig. 1B). Activation of PO_4Akt can be abrogated by pre-treatment with the selective PI-3-kinase inhibitor, LY294002 (50 μM) (Fig. 1B).

3.2. BDNF rescues NB cells from cisplatin-induced apoptosis

BDNF pre-treatment of retinoic-acid treated NB cells has been demonstrated to rescue cells from vinblastine-induced cytotoxicity [8]. Recently we showed that BDNF via activating the TrkB path through PI-3-kinase also protects cells from etoposide cytotoxicity [10]. Cisplatin is an important chemotherapeutic agent in the treatment of NB. In this study, we investigate the ability of BDNF to protect two different TrkB-expressing cells from cisplatin-induced cytotoxicity. BDNF does not have a significant effect on the proliferation of control cells [7]. In order to determine the effect of cisplatin on the viability of NB cells, we treated the cells with different concentrations of cisplatin (0–100 μg/ml) for 2 h, and then incubated the cells with untreated media. After 48 h, relative cell number was assessed by using an MTT assay (Fig. 2A,B). Treatment of the AS-TB8 and SY5Y-TB8 cells with chemotherapy results in a dose-responsive cell death as measured by MTT (Fig. 2A,B). Pre-treatment of these cells with BDNF (100 ng/ml) for 15 min prior to addition of cisplatin shows a significant protection from cisplatin-induced cytotoxicity at the indicated drug doses (Fig. 2A,B). Both AS and SY5Y cells treated with BDNF required almost 5-fold more Cisplatin to reach the IC50.

3.3. Inhibition of the Trk tyrosine kinase and PI-3-kinase pathways

In order to show the dependence of the BDNF rescue of cisplatin on TrkB, we pre-treated the cells with the selective Trk kinase inhibitor, K252a (500 nM). K252a was incubated for 30 min prior to addition of BDNF (100 ng/ml). Following BDNF treatment, the cells were treated with either 5 μg/ml cisplatin (AS-TB8) or 10 μg/ml (SY5Y-TB8). After 2 h, cells were washed and cultured for 48 h with media alone. Pre-treatment with K252a results in complete abrogation of BDNF rescue of both the AS-TB8 and SY5Y-TB8 cell lines from cisplatin-induced cytotoxicity (Fig. 3A,
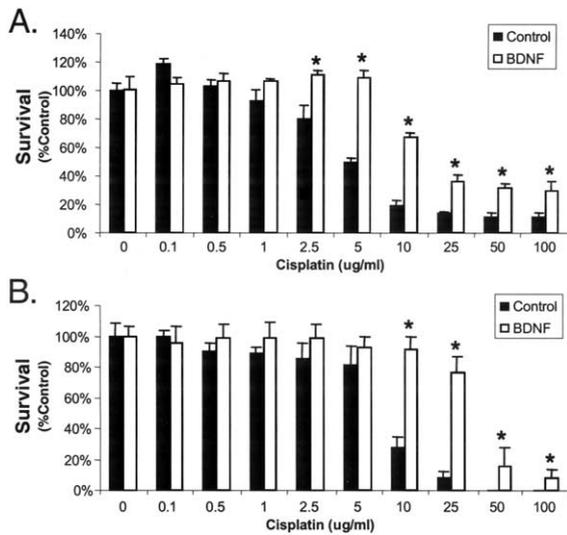
Fig. 2. BDNF rescues TrkB-expressing NB cells from cisplatin-induced cytotoxicity. (Panels A and B) MTT analysis of AS-TB8 and SY5Y-TB8 cells in indicated conditions. Cells were cultured in complete medium for 16–24 h. On the 3rd day, cells were stimulated with BDNF (100 ng/ml) for 15 min where indicated. Cells were then treated with varying concentrations of cisplatin (0–100 μg/ml) for 2 h. Media was replaced, and cells were cultured for an additional 48 h prior to MTT analysis. (Panel A) AS-TB8 cells; (Panel B) SY5Y-TB8 cells. * indicates P value < 0.01.

B), demonstrating that rescue is dependent on TrkB activation. Nerve growth factor (NGF), which binds the p75 receptor, but not TrkB fails to rescue cells from cisplatin under these conditions (data not shown).

To determine the role of the PI3-kinase pathway in the BDNF-mediated rescue from cisplatin, we used the PI-3-kinase inhibitor, LY294002. AS-TB8 and SY5Y-TB8 cells were pre-treated with LY294002 (50 μM) and culture as above. LY294002 abrogated the BDNF rescue of cisplatin-induced cytotoxicity, indicating that the PI-3-kinase pathway is primarily responsible for BDNF rescue. Incubation with pharmacologic inhibitors of the mitogen-activated protein kinase (MAPK) pathway (PD98059) and the PLCγ pathway (U73122) failed to block BDNF rescue from cisplatin induced cell death (data not shown).

4. Discussion

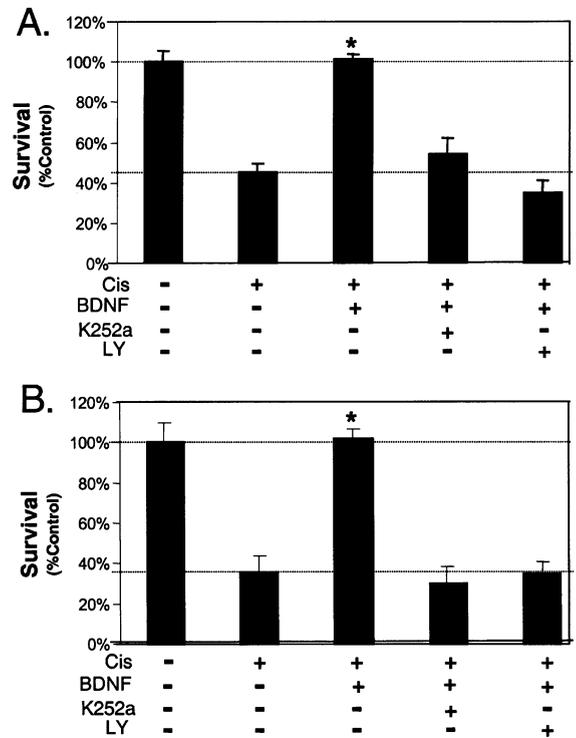Neurotrophins have the ability to protect normal

Fig. 3. Pharmacologic blockade of Trk kinase activity and PI3-kinase activity abrogates BDNF rescue. (Panels A and B) K252a and LY294002 abrogate BDNF rescue. Cells were cultured in complete medium for a period of 3 days. On the 3rd day, the cells were treated with or without K252a (500 nM) or LY294002 (50 μM) for 30 min, followed by BDNF (100 ng/ml) for a period of 15 min. Then cells were treated with either 5 μg/ml cisplatin (AS-TB8) or 10 μg/ml cisplatin (SY5Y-TB8) for 2 h. Subsequently, the media was replaced with untreated media and the cells were incubated for 48 h. (Panel A) AS-TB8 cells; (Panel B) SY5Y-TB8 cells. * indicates P value < 0.01.

neurons from both chemical and physical injuries [19]. We have hypothesized that the expression of BDNF and TrkB by neuroectodermal tumors may contribute to chemoresistance [8]. Several studies have shown that neurotrophins, particularly BDNF, can promote the survival [2,4,5] and chemoresistance of neuroblastoma cells, however the mechanism of this chemoprotection has yet to be elucidated. In this study, we demonstrate that BDNF is capable of rescuing NB cells from cisplatin-induced cell death. In addition, we have determined that the BDNF rescue can be inhibited by pre-treatment with either a Trk kinase or PI-3-kinase inhibitor.

We have found that BDNF blocks cisplatin, as well

as etoposide, doxorubicin and vinblastine [10], through activation of TrkB. This is evident in that the selective Trk tyrosine kinase inhibitor, K252a, is able to prevent rescue by BDNF. Further evidence lies in the fact that both parental cell lines are unable to be rescued by BDNF treatment (data not shown). One NB model has shown that NGF can mediate protection from neocarzinostatin-induced apoptosis by a p75 dependent mechanism [20]. However the role of p75 signaling can be confusing in that it has been known to have both pro- and anti-apoptotic signaling capacities, and the ability of p75 to rescue is dependent on the levels of Trk receptors present [21].

Using a pharmacologic approach, we have also shown that inhibition of the PI-3 kinase path is able to prevent BDNF rescue. In neurons, various signaling paths have been implicated in rescue from ischemic injury. For example, in neonatal brains BDNF rescue from hypoxic-ischemic injury is dependent on activation of the MAPK pathway [22]. In oxidative stress-induced [23] and axotomy-induced [24] cell death, cerebellar granule neurons and retinal ganglion cells, respectively, are rescued by activation of both the PI-3 kinase and MAPK pathways.

Our studies would indicate that chemotherapy in combination with a PI-3-kinase inhibitor could rescue from BDNF induced chemoresistance. We believe the implication of this study for treatment of aggressive and chemoresistant NB tumors is important. CEP751, a K252a derivative with greater selectivity for the Trk kinase, has already been shown to inhibit growth in TrkB-expressing neuroblastoma xenografts in nude mice [25]. Pharmacologic targeting of the PI-3 kinase path in NB represents an alternative strategy with potentially fewer or different toxicities when combined with chemotherapy to treat NB tumors.

Acknowledgements

References

[1] G. Brodeur, J. Pritchard, F. Berthold, N. Carlsen, V. Castel, Revision of the international criteria for neuroblastoma diagnosis, staging and response to treatment, J. Clin. Oncol. 11 (1993) 1466–1477.

[2] A. Nakagawara, M. Arima-Nakagawara, N. Scavarda, C. Azar, A. Cantor, G. Brodeur, Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma, N. Engl. J. Med. 328 (1993) 847–854.

[3] G.M. Brodeur, A. Nakagawara, D.J. Yamashiro, N. Ikegaki, X-G. Liu, C.G. Azar, C.P. Lee, A.E. Evans, Expression of trkA, trkB, and trkC in human neuroblastoma, J. Neuro. Oncol. 31 (1997) 49–55.

[4] K. Matsumoto, R.K. Wada, J.M. Yamashiro, D.R. Kaplan, C.J. Thiele, Expression of brain-derived neurotrophic factor and p145trkB affects survival, differentiation, and invasiveness of human neuroblastoma cells, Cancer Res. 55 (1995) 1798–1806.

[5] X. Feng, H. Jiang, J.C. Baik, C. Edgar, F.F. Eide, BDNF dependence in Neuroblastoma, J. Neurosci. Res. 64 (2001) 355–363.

[6] D.R. Kaplan, K. Matsumoto, E. Lucarelli, C.J. Thiele, Induction of trkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells, Neuron 11 (1993) 321–331.

[7] A. Nakagawara, C. Azar, N. Scavarda, G. Brodeur, Expression and function of TRK-B and BDNF in human neuroblastoma, Mol. Cell Biol. 14 (1994) 759–767.

[8] S. Scala, K. Wosikowski, P. Giannakakou, P. Valle, J.L. Biedler, B.A. Spengler, E. Lucarelli, S.E. Bates, C.J. Thiele, Brain-derived neurotrophic factor protects neuroblastoma cells from vinblastine toxicity, Cancer Res. 56 (1996) 3737–3742.

[9] D.S. Middlemas, B.K. Kihl, J. Zhou, X. Zhu, Brain-derived neurotrophic factor promotes survival and chemoprotection of human neuroblastoma cells, J. Biol. Chem. 274 (1999) 16451–16460.

[10] J. Jaboin, C.J. Kim, D.R. Kaplan, C.J. Thiele, BDNF activation of TrkB protects neuroblastoma cells from chemotherapy-induced apoptosis via PI-3 kinase, Cancer Res. 62 (2002) 6756–6763.

[11] R. Ventimiglia, P. Mather, B. Jones, R. Lindsay, The neurotrophins BDNF, NT-3 and NT-4/5 promote survival and morphological and biochemical differentiation of striatal neurons in vitro, Eur. J. Neurosci. 7 (1995) 213–222.

[12] A. Acheson, J. Conover, J. Fandl, T. DeChiara, M. Russel, A. Thadani, S. Squinto, G. Yancopoulos, R. Lindsay, A BDNF autocrine loop in adult sensory neurons prevents cell death, Nature 30 (1995) 405–406.

[13] S. Alcantara, J. Frisen, J.A. del Rio, E. Soriano, M. Barbacid, I. Silos-Santiago, TrkB signaling is required for postnatal survival of CNS neurons and protects hippocampal and motor neurons from axotomy-induced cell death, J. Neurosci. 17 (1997) 3623–3633.

[14] B. Cheng, M.P. Mattson, NT-3 and BDNF protect CNS

neurons against metabolic/excitotoxic insults, Brain Res. 640 (1994) 56–67.

[15] D. Lindholm, G. Dechant, C. Heinsenberg, H. Thoenen, Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity, Eur. J. Neurosci. 5 (1993) 1455–1464.

[16] S. Shimohama, Y. Tamura, A. Akaike, T. Tsukahara, O. Ohara, S. Watanabe, J. Kimura, Brain-derived neurotrophic factor pretreatment exerts a partially protective effect against glutamate-induced neurotoxicity in cultured rat cortical neurons, Neuroscience 164 (1993) 55–58.

[17] M.J. Courtney, K.E. Akerman, E.T. Coffey, Neurotrophins protect cultured cerebellar granule neurons against the early phase of cell death by a two-component mechanism, J. Neurosci. 17 (1997) 4201–4211.

[18] C.J. Kim, T. Matsuo, K.H. Lee, C.J. Thiele, Up-regulation of insulin growth factor-II expression is a feature of TrkA but not TrkB activation in SH-SY5Y neuroblastoma cells, Am. J. Pathol. 155 (1999) 1661–1670.

[19] O. Lindvall, Z. Kokaia, J. Bengzon, E. Elmer, M. Kokaia, Neurotrophins and brain insults, Trends Neurosci. 17 (1994) 490–496.

[20] M.H. Cortazzo, E.S. Kassis, K.A. Sproul, N.F. Schor, Nerve growth factor (NGF)-mediated protection of neural crest cells from antimitotic agent-induced apoptosis: the role of the low-affinity NGF receptor, J. Neurosci. 16 (1996) 3895–3899.

[21] F.D. Miller, D.R. Kaplan, Neurotrophin signalling pathways regulating neuronal apoptosis, Cell Mol. Life Sci. 58 (2001) 1045–1053.

[22] B.H. Han, D.M. Holtzman, BDNF protects the neonatal brain from hypozix-ischemic injury in vivo via the ERK pathway, J. Neurosci. 20 (2000) 5775–5781.

[23] S.D. Skaper, M. Floreani, A. Negro, L. Facci, Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway, J. Neurochem. 70 (1998) 1859–1868.

[24] N. Klocker, P. Kermer, J.H. Weishaupt, M. Labes, R. Ankerhold, M. Bahr, Brain-derived neurotrophic factor-mediated neuroprotection of adult rat retinal ganglion cells in vivo does not exclusively depend on phosphatidyl-inositol-3'-kinase/protein kinase B signaling, J. Neurosci. 20 (2000) 6962–6967.

[25] A.E. Evans, K.D. Kisselbach, X. Liu, A. Eggert, N. Ikegaki, A.M. Camoratto, C. Dionne, G.M. Brodeur, Effect on CEP-751 (KT-6587) on neuroblastoma xenografts expressing TrkB, Med. Pediatr. Oncol. 36 (2001) 181–184.