Inactivation of glycogen synthase kinase-3β contributes to brain-derived neurotrophic factor/TrkB-induced resistance to chemotherapy in neuroblastoma cells

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Abstract

Elucidating signaling pathways that mediate cell survival or apoptosis will facilitate the development of targeted therapies in cancer. In neuroblastoma tumors, brain-derived neurotrophic factor (BDNF) and its receptor TrkB are associated with poor prognosis. Our previous studies have shown that BDNF activation of TrkB induces resistance to chemotherapy via activation of phosphoinositide-3-kinase (PI3K)/Akt pathway. To study targets of PI3K/Akt that mediate protection from chemotherapy, we focused on glycogen synthase kinase-3β (GSK-3β), which is a known modulator of apoptosis. We used pharmacologic and genetic methods to study the role of GSK-3β in the BDNF/TrkB/PI3K/Akt protection of neuroblastoma from chemotherapy. BDNF activation of TrkB induces the Akt-dependent phosphorylation of GSK-3β, resulting in its inactivation. Treatment of neuroblastoma cells with inhibitors of GSK-3β, LiCl, GSK-3β inhibitor VII, kenpaullone, or a GSK-3β-targeted small interfering RNA (siRNA) resulted in a 15% to 40% increase in neuroblastoma cell survival after cytotoxic treatment. Transfection of neuroblastoma cells with a constitutively active GSK-3β S9A9 caused a 10% to 15% decrease in cell survival. Using real-time, dynamic measurements of cell survival, we found that 6 to 8 h after etoposide treatment was the period during which critical events regulating the induction of cell death or BDNF/TrkB-induced protection occurred. During this period, etoposide treatment was associated with the dephosphorylation and activation of GSK-3β in the mitochondria that was blocked by BDNF activation of TrkB. These data indicate that the inactivation of GSK-3β contributes to the BDNF/TrkB/PI3K/Akt protection of neuroblastoma cells from chemotherapy. [Mol Cancer Ther 2007;6(12):3113–21]

Introduction

Neuroblastoma is a common solid tumor in children that derives from neural crest precursor cells (1). The expression of brain-derived neurotrophic factor (BDNF) and its receptor TrkB (2, 3) are more highly expressed in tumors from patients who have a poor prognosis. In vitro studies indicate that BDNF activation of TrkB protects neuroblastoma cells from chemotherapy (4–6). BDNF activation of TrkB can initiate several signaling pathways, including phosphoinositide-3-kinase (PI3K), mitogen-activated protein kinase, and phospholipase C-γ (PLC-γ) pathways (7), and our previous studies established that BDNF activation of TrkB via the PI3K/Akt signaling pathway attenuates the effects of chemotherapy in neuroblastoma cells (8, 9). Moreover, we found that constitutive activation of Akt in the absence of growth factors alters the sensitivity of cells to cytotoxic drugs used in the therapy of neuroblastoma (9). To define the signaling pathways downstream of PI3K/Akt that mediate resistance to chemotherapy, we investigated whether glycogen synthase kinase-3β (GSK-3β) modulates the sensitivity of neuroblastoma cells to chemotherapy.

GSK-3β is a ubiquitously expressed serine/threonine kinase in mammals and generally considered constitutively active in resting cells. The most well-defined post-translational modification of GSK-3β is phosphorylation of Ser9, which inhibits its activity (10, 11). GSK-3β was initially identified as an enzyme that regulates glycogen synthesis in response to insulin. Insulin binds its tyrosine kinase receptor and activates PI3K/Akt signaling, which results in the phosphorylation and inactivation of GSK-3β (12). GSK-3β is a major regulator of the Wnt signaling pathway, where activation of GSK-3β determines cell fate in embryonic development (13). However, GSK-3β also mediates cell survival or apoptosis in different cell types in response to different stimuli (14–19). A role for GSK-3β in regulating apoptosis downstream of PI3K/Akt signaling pathway was first shown in Rat-1 fibroblasts and PC12 pheochromocytoma cells (14). Activation of GSK-3β also promotes apoptosis induced by DNA damage, heat shock, staurosporine, and endoplasmic reticulum (ER) stress (15–19). These findings indicate that GSK-3β is an important intracellular signaling molecule that modulates cell death and survival signals.

Neurotrophic factors are necessary for the survival and function of neurons. BDNF exerts its initial action by interacting with TrkB receptors to initiate intracellular signaling pathways. Our previous studies identified that
BDNF activation of TrkB protects neuroblastoma cells from chemotherapy predominantly via the activation of the PI3K/Akt signal transduction pathway (8, 9). GSK-3β is a substrate of Akt, so in this study, we evaluated the role of GSK-3β in mediating the BDNF/TrkB/PI3K/Akt-induced survival signals in response to cytotoxic drugs in neuroblastoma cells. We find that the inactivation of GSK-3β by BDNF/TrkB/Akt attenuates the sensitivity of neuroblastoma cells to chemotherapy.

Materials and Methods

Cells and Cell Culture

Stable TrkB-expressing cell line TB8 and NGP-TrkB cells (20) were cultured in RPMI 1640 (Mediatech, Inc.) containing 10% fetal bovine serum (FBS), 2 mmol/L glutamine, and antibiotics at 37°C in 5% CO2 incubator. To maintain TrkB selection, neuroblastoma cells were cultured in puromycin (0.5 μg/mL; Sigma-Aldrich Inc.).

DNA Vector Constructions

The GSK-3β (S9A9) plasmid (21) was used in this study, and a constitutively active Akt and empty vector (EV) were provided by Dr. M.J. Quon (Hypertension-Endocrine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) (22).

Reagents

Recombinant human BDNF was from PeproTech, Inc. Akt inhibitor SH-6, GSK-3β inhibitor VII, and kempaulone were purchased from Calbiochem. Etoposide, cisplatin, and lithium chloride were obtained from Sigma. Anti-phospho–GSK-3β (Ser9) and anti-histone were obtained from Cell Signaling Technology, and anti–GSK-3β was from BD Transduction Laboratories. Anti-tubulin and anti-actin antibodies were from Santa Cruz Biotechnology, and anti-hemagglutinin (HA) and anti-cyclooxygenase IV (anti-COX IV) antibodies were from Abcam Inc.

Transfection

GSK-3β small interfering RNA (siRNA) or siRNA control (Santa Cruz Biotechnology), constitutively active GSK-3β, constitutively active Akt or EV were transected into TB8 (2 × 106) cells by the Nucleofector device from Amaxa Biosystems using cell line Nucleofector Kit V and program A-023 for TB8 cells and program A-030 for NGP-TrkB cells.

Western Blotting

Subcellular proteins were extracted according to the instruction of the Fractionation Kit (Active Motif); whole cell proteins were extracted as previously reported (8). Western blotting analyses were done as described previously (8). Briefly, protein lysate (30 μg) was analyzed by SDS-PAGE and transferred to nitrocellulose. The indicated primary antibodies were diluted according to manufacturer’s recommendations and detected with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse immunoglobulin G (1:2,000 dilution).

Treatments

Neuroblastoma cells were cultured in RPMI 1640 containing 10% FBS for 24 h and treated with BDNF (100 ng/mL) for the indicated times. Cells were treated with lithium chloride at 20 mmol/L for the indicated times or for 24 h at the indicated concentrations. To study the inhibitory effect of Akt inhibitor SH-6, cells were first cultured in serum-free medium for 16 h, treated with SH-6 for 1 h, followed by BDNF (1 h, 100 ng/mL) stimulation. To study the cell survival after treatment with chemotherapeutic drugs, cells were pretreated with GSK-3β inhibitors or transfected with GSK-3β siRNA or constitutively active GSK-3β (S9A9) plasmid, followed by treatment with etoposide or cisplatin for 24 h.

Cell Survival Analysis

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay (MTS assay; Promega Corporation) was done according to the manufacturer’s specification. The percentage of cell survival (% survival) was calculated by dividing the absorbance value of the treated samples by the absorbance value of the untreated control within every group. All experiments were repeated twice to thrice.

Assay of GSK-3β Activity

The activity of GSK-3β was assayed using an immune complex in vitro kinase assay by the methods of Fang (23) and Kuemmerle (24). Briefly, neuroblastoma cells were first treated differently, and then proteins were extracted, and GSK-3β was immunoprecipitated. Kinase activity of immunoprecipitated GSK-3β was assayed using phosphoglycogen synthase peptide-2 as substrate or glycogen synthase peptide-2[Ala21] as a negative control peptide. The values of treated groups were normalized by the value of control group.

Real-time Cell Electronic Sensing System

The principles of the real-time cell electronic sensing (RT-CES) system (ACEA Biosciences Inc.) have been described previously (25, 26). Briefly, The RT-CES system used impedance sensor technology to noninvasively quantify cells in real time based on cell number, morphology, and adhesion. By using the RT-CES system, changes in the dynamic cellular status induced by chemotherapeutic drugs were monitored. Cell proliferation increases the cell index (CI) value, whereas cell death or cytotoxicity induced cell detachment, resulting in a lower CI value. TB8 cells were seeded into the ACEA E-plate at a density of 40,000 cells per well, and cell growth and proliferation were dynamically monitored using the RT-CES system. Twenty-four hours after seeding, BDNF (100 ng/mL, 1 h) was added followed by etoposide (1 μg/mL) treatment, and TB8 cell viability was dynamically monitored.

Cell Fractionation

Mitochondria and cytosolic fractions were prepared using a mitochondria fractionation kit (40015); nuclear fractions were prepared using a nuclear extract kit (40010), purchased from Active Motif. Cells were first treated according to the experimental design, and then subcellular fractions were extracted according to manufacturer’s specifications.

Mitochondrial Membrane Potential Measurement-JC-1 Flow Cytometry

To measure the mitochondrial membrane potential, neuroblastoma cells were stained with fluorescent probe JC-1 (5,5′,6,6′-tetrachloro-1′,3′,3′-tetrabutylbenzimidazol...
BDNF activation of TrkB induces Akt-dependent phosphorylation of GSK-3β

We have previously reported that as early as 5 min after BDNF activation of TrkB, increased Akt phosphorylation at Ser\(^{473}\) is detected in neuroblasts (9). GSK-3β is one of the key effectors of Akt signaling. To test whether BDNF/TrkB-induced Akt activation phosphorylates GSK-3β in neuroblasts, TB8 cells, which express high TrkB levels, were treated with BDNF (100 ng/mL) for varying times (0.5, 1, 3, and 6 h). Western blots of whole cell protein lysates were probed with antibodies specific for phosphorylated GSK-3β (Ser\(^{9}\)) and total GSK-3β. Maximal (4-fold) phosphorylation of GSK-3β (Ser\(^{9}\)) [P-GSK-3β(Ser\(^{9}\))] was detected within 30 min of BDNF treatment, and levels of P-GSK-3β(Ser\(^{9}\)) remained elevated (2-fold) at all time points tested (within 6 h). There was no change in the absolute levels of GSK-3β (T-GSK-3β) after BDNF stimulation (Fig. 1A).

Because previous studies have shown that GSK-3β is inactivated by phosphorylation at the NH\(_2\)-terminal Ser\(^{9}\) (11), we assessed whether BDNF/TrkB-induced phosphorylation of GSK-3β resulted in the inhibition of GSK-3β activity in our model system. BDNF activation of TrkB caused a 50% decrease in the ability of GSK-3β to phosphorylate its substrate glycogen synthase, and there was a persistent 40% decrease in GSK-3β kinase activity even after 6 h of BDNF treatment (Fig. 1B). The decrease in GSK-3β kinase activity was consistent with the persistent increase in P-GSK-3β(Ser\(^{9}\)) (Fig. 1A). These data indicated that BDNF activation of TrkB induced the phosphorylation of GSK-3β that resulted in a reduction in the kinase activity of GSK-3β.

To determine whether BDNF/TrkB regulation of GSK-3β was Akt dependent, TB8 cells were transfected with constitutively active Akt or treated with SH-6, a pharmacologic inhibitor of Akt, before BDNF stimulation. Transfection of constitutively active Akt into TB8 cells induced an increase of GSK-3β phosphorylation, but did not affect T-GSK-3β level (Fig. 1C). Pretreatment of TB8 cells with SH-6 before BDNF administration blocked the BDNF-induced phosphorylation of GSK-3β, but did not alter T-GSK-3β levels (Fig. 1D). These results indicated that Akt mediated the BDNF/TrkB regulation of GSK-3β phosphorylation in TB8 cells.

Pharmacologic inhibition of GSK-3β attenuates the cytotoxic effects of etoposide and cisplatin on neuroblastoma cells

We have previously reported that BDNF/TrkB-induced activation of Akt mediates the BDNF/TrkB protection of neuroblastoma cells from chemotherapy (9). Because either BDNF activation of TrkB or expression of a constitutively active Akt induced the phosphorylation and inactivation of GSK-3β (Fig. 1), we sought to determine whether the inactivation of GSK-3β would also protect neuroblastoma cells from chemotherapy. TB8 cells were treated with lithium chloride (LiCl) at different doses for 24 h or 20 mmol/L for different times. LiCl is a drug used for the treatment of bipolar disease, which is also recognized as a GSK-3β inhibitor (27). Treatment with 20 mmol/L LiCl caused a 50% decrease in the ability of GSK-3β to phosphorylate its substrate glycogen synthase, and there was a persistent 40% decrease in GSK-3β kinase activity even after 6 h of BDNF treatment (Fig. 1B). The decrease in GSK-3β kinase activity was consistent with the persistent increase in P-GSK-3β(Ser\(^{9}\)) (Fig. 1A). These data indicated that BDNF activation of TrkB induced the phosphorylation of GSK-3β that resulted in a reduction in the kinase activity of GSK-3β.

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![Figure 1](https://example.com/figure1.png)
resulted in an increase in GSK-3β phosphorylation that occurred within 1 h and peaked at 24 h (Fig. 2A). Similar results were also found when another TrkB-expressing cell line, NGP-TrkB, was tested (Fig. 2B). These data indicated that LiCl induced the GSK-3β phosphorylation.

To study the role of GSK-3β in cell survival, we pretreated the TB8 (24 h) or NGP-TrkB (2 h) cells with 20 mmol/L LiCl, followed by etoposide or cisplatin for 24 h. There was a 15% or 25% increase of cell survival in TB8 cells (Fig. 2C) in LiCl pretreated cells followed by etoposide or cisplatin treatment, respectively. Similar results were observed in NGP-TrkB cells (Fig. 2D). These data indicated that LiCl pretreatment resulted in a statistically significant increase in the survival of neuroblastoma cells from chemotherapy-induced cell death.

To confirm the contribution of GSK-3β inhibition to chemoresistance, neuroblastoma cells were pretreated with another two GSK-3β inhibitors, GSK-3β inhibitor VII and

Figure 2. GSK-3β inhibitors increased neuroblastoma cell survival following chemotherapy. A and B, neuroblastoma cells (TB8 cells in A, NGP-TrkB cells in B) were treated with LiCl (0.5, 1, 2, 5, 10, 20, and 30 mmol/L) for 24 h or at 20 mmol/L for the indicated times (0.5, 1, 3, 12, 24, and 36 h in [A] or 2, 8, 16, and 24 h in [B]), and total proteins were extracted and analyzed for P-GSK-3β(Ser9) and T-GSK-3β by Western blotting. C and D, neuroblastoma cells were treated with LiCl at 20 mmol/L for 24 h in TB8 cells (C) or 2 h in NGP-TrkB cells (D), followed by treatment with etoposide (1 μg/mL in TB8 cells (C), 10 μg/mL in NGP-TrkB cells (D)) or cisplatin (3 μg/mL in TB8 cells (C), 20 μg/mL in NGP-TrkB cells (D)). E, TB8 cells were pretreated with GSK-3β inhibitor VII (7.5 μmol/L) or kenpaullone (15 μmol/L) for 3 h, followed by the treatment of etoposide (1 μg/mL) or cisplatin (3 μg/mL) for 24 h. F, NGP-TrkB cells were pretreated with GSK-3β inhibitor VII (10 μmol/L) or kenpaullone (20 μmol/L) for 3 h, followed by the treatment of etoposide (10 μg/mL) or cisplatin (20 μg/mL) for 24 h. MTS assay was used to detect the cell survival. Survival rate in every condition was normalized to the no-etoposide or no-cisplatin groups. Bars, SD. *, P < 0.05; **, P < 0.01, versus etoposide-treated groups; †, P < 0.05; ‡, P < 0.01, versus cisplatin-treated groups. G and H, TB8 cells (G) or NGP-TrkB cells (H) were treated with LiCl, GSK-3 inhibitor VII or kenpaullone as described in (C and E) or (D and F), respectively, and then proteins were extracted and assessed using an in vitro kinase assay to determine the GSK-3β kinase activity.
respectively (Fig. 3C). In NGP-TrkB cells, the survival of transfected cells following etoposide or cisplatin treatment, cells was 13% or 14% higher than that of the control siRNA-transfected cells, or cells were treated with etoposide (1 μg/mL in C; 10 μg/mL in D) or cisplatin (3 μg/mL in C; 20 μg/mL in D) for 24 h, and MTS assay was used to detect the cell survival. Survival rate in every group was normalized to the untreated control. *, *P < 0.05 (in etoposide-treated cells), versus control siRNA-transfected cells; #, #P < 0.01, versus control siRNA-transfected cells.

Figure 3. Knockdown of GSK-3β by siRNA increased the cell survival following chemotherapy. Neuroblastoma cells were cultured for 24 h and then transfected with GSK-3β siRNA (2 μg) or control siRNA (2 μg) using an Amaxa machine, program A-023 for TB8 cells (A and C) or program A-030 for NGP-TrkB cells (B and D). Forty-eight hours after transfection, proteins were analyzed for T-GSK-3β and tubulin or for GSK-3β activity by in vitro kinase assay [A and B; *, *P < 0.05; **, **P < 0.01, versus control siRNA-transfected cells] or cells were transfected with etoposide (1 μg/mL in C; 10 μg/mL in D) or cisplatin (3 μg/mL in C; 20 μg/mL in D) for 24 h, and MTS assay was used to detect the cell survival. Survival rate in every group was normalized to the untreated control. *, *P < 0.05 (in etoposide-treated cells), versus control siRNA-transfected cells; #, #P < 0.01, versus control siRNA-transfected cells.

kenpaullone. GSK-3β inhibitor VII is a non-ATP-competitive inhibitor of GSK-3β, whereas kenpaullone is an ATP-competitive inhibitor of GSK-3β (28). Neuroblastoma cells were pretreated with GSK-3β inhibitor VII for 3 h at 7.5 μmol/L or with kenpaullone for 3 h at 15 μmol/L and then treated with etoposide or cisplatin for 24 h. In TB8 cells, pretreatment with GSK-3β inhibitor VII increased the cell survival by 20% or 28% following etoposide or cisplatin treatment, respectively; the increase of cell survival was 22% or 36% following etoposide or cisplatin treatment, respectively, when the cells were pretreated with kenpaullone (Fig. 2E). In NGP-TrkB cells, pretreatment with GSK-3β inhibitor VII increased cell survival by 40% or 30% following etoposide or cisplatin treatment, respectively. The increase of cell survival was 40% or 45% following etoposide or cisplatin treatment, respectively, when the cells were pretreated with kenpaullone (Fig. 2F). In vitro kinase assay showed that LiCl, GSK-3β inhibitor VII, and kenpaullone inhibited GSK-3β kinase activity in these two cell lines (Fig. 2G and H). These data indicate that pretreatment of neuroblastoma cells with GSK-3β inhibitors protected neuroblastoma cells from chemotherapy-induced cell death.

Knockdown of GSK-3β by siRNA Increases Cell Survival Following Chemotherapy

We used the gene silencing method to study the role of GSK-3β in cell survival. We transfected GSK-3β siRNA and control siRNA into neuroblastoma cells and found that after 48 h, there was a 65% or 70% decrease of GSK-3β protein levels in TB8 cells (Fig. 3A) or NGP-TrkB cells (Fig. 3B), respectively. The GSK-3β activity decreased simultaneously in these two cell lines. Forty-eight hours following GSK-3β or control siRNA transfection, cells were treated with etoposide or cisplatin for an additional 24 h. In TB8 cells, the survival of GSK-3β siRNA-transfected cells was 13% or 14% higher than that of the control siRNA-transfected cells following etoposide or cisplatin treatment, respectively (Fig. 3C). In NGP-TrkB cells, the survival of GSK-3β siRNA-transfected cells was 16% or 15% higher than that of the control siRNA-transfected cells following etoposide or cisplatin treatment, respectively (Fig. 3D). These differences were statistically significant. These data indicated that knockdown of GSK-3β by siRNA attenuated the effects of chemotherapy on neuroblastoma cells.

Constitutively Active GSK-3β Increases the Sensitivity of Neuroblastoma Cells to Chemotherapy

To further examine the role of GSK-3β in cell survival, we transfected a constitutively active HA-tagged GSK-3β plasmid (S9A9) into neuroblastoma cells. The S9A9 plasmid contains a mutation of Ser9, so that it cannot be phosphorylated, and thus, GSK-3β maintains an active conformation. Forty-eight hours after transfection with S9A9 or a control EV plasmid, the neuroblastoma cells were harvested. The transfected GSK-3β was detected by HA antibody in the S9A9-transfected cells in both TB8 (Fig. 4A) and NGP-TrkB cells (Fig. 4B). There was a non-specific band in the EV-transfected cells when immunoblotted by HA antibody. The native T-GSK-3β level was the same between EV-transfected cells and S9A9 transfected in the two cell lines (Fig. 4A and B). P-GSK-3β(Ser9) levels were found 1.7-fold or 1.5-fold decrease in S9A9-transfected cells compared with EV-transfected cells in TB8 cells (Fig. 4A) or NGP-TrkB cells (Fig. 4B), respectively. In vitro kinase assay showed that GSK-3β activity increased in S9A9-transfected cells compared with EV-transfected cells in each of the cell lines (Fig. 4A and B). To study the effects of active GSK-3β on cell survival, neuroblastoma cells were treated with etoposide or cisplatin for an additional 24 h after transfection of S9A9 or EV, and cell survival was assessed. In TB8 cells, the survival of S9A9-transfected cells was 16% or 14% lower than that of the EV-transfected cells following etoposide or cisplatin treatment, respectively (Fig. 4C). In NGP-TrkB cells, the survival of S9A9-transfected cells was 15% or 13% lower than that of the EV-transfected cells following etoposide or cisplatin treatment, respectively (Fig. 4D). No decrease of cell...
survival was detected in S9A9-transfected cells compared with EV-transfected cells in the absence of etoposide or cisplatin. These data indicated that constitutively active GSK-3β increased the sensitivity of neuroblastoma cells to etoposide and cisplatin.

**Knockdown of GSK-3β Attenuates Etoposide-Induced Decrease of Mitochondrial Membrane Potential**

To dynamically monitor the changes in the cells after etoposide treatment, we used the RT-CES system. TB8 cells were pretreated with BDNF (100 ng/mL, 1 h) and then treated with etoposide. In Fig. 5A, the CI values in control cells and BDNF-treated cells continued to increase with time in culture, and no statistical difference was detected. Etoposide treatment decreased the CI value of the TB8 cells and pretreatment with BDNF before etoposide attenuated the etoposide-induced decrease of the CI value. At the end of this experiment, we used the cells to perform a MTS assay, and the result was consistent with the RT-CES data (Fig. 5B). The results from the RT-CES system indicated that critical events regulating cell survival or death signals occurred during this time. To investigate if critical events regulating cell survival or death of cells occurred 6 to 8 h after etoposide treatment (Fig. 5A). For this reason, we extracted proteins from the nuclear, cytosolic, and mitochondrial compartments in TB8 cells at 8 and 16 h after etoposide treatment. As early as 8 h after etoposide treatment, there was a decrease in GSK-3β phosphorylation in the mitochondrial fraction, whereas levels in the nuclear and cytosolic compartments did not show significant changes at this time, but declined by 16 h (Fig. 6B). This etoposide-induced decrease in GSK-3β phosphorylation in the mitochondria was blocked by the pretreatment of BDNF before etoposide (Fig. 6C). To investigate whether BDNF protects neuroblastoma cells from chemotherapy via mitochondria, we analyzed the changes of mitochondrial membrane potential after different treatment by flow cytometry analysis using the fluorescent probe JC-1. JC-1 is selectively taken up by the mitochondria and is a reliable indicator of change in the mitochondrial membrane potential. In control cells, the scattergram showed that most cells presented in the R2 region with high red fluorescence (FL-2) and high green fluorescence (FL-1), which revealed a normal mitochondrial membrane potential. Twelve percent of cells were in the R4 region, which had low red fluorescence (FL-2), indicating depolarization.

**Figure 4.** Constitutively active GSK-3β increased the sensitivity of neuroblastoma cells to chemotherapy. Neuroblastoma cells were cultured 24 h and then transfected with constitutively active GSK-3β (S9A9) or EV using an Amaxa machine, program A-023 for TB8 cells (A and C) or program A-030 for NGP-TrkB cells (B and D). Forty-eight hours after transfection, whole cell lysate was extracted, and proteins were analyzed for HA, T-GSK-3β, and tubulin or for GSK-3β activity by in vitro kinase assay (A and B). *, P < 0.05, versus EV-transfected cells, or cells were treated with etoposide (0.5 μg/mL in C; 5 μg/mL in D) or cisplatin (1 μg/mL in C; 10 μg/mL in D) for 24 h, and MTS assay was used to detect the cell survival. Survival rate in every group was normalized to the untreated control. *, P < 0.05 (in etoposide-treated cells), versus EV-transfected cells; #, P < 0.05 (in cisplatin-treated cells), versus EV-transfected cells.

**Figure 5.** A. Three hours after treatment, the RT-CES system was used to analyze the subcellular fractionation of cells and extracted proteins from the nuclear, cytosolic, and mitochondrial compartments. Histone, actin, and COX IV were used to monitor the purity of the proteins in the nuclear, cytosolic, and mitochondrial compartments, respectively (Fig. 6A, left). T-GSK-3β was detected in the nuclear, cytosolic, and mitochondrial compartments of the TB8 cells, and BDNF (100 ng/mL, 15 and 60 min) activation of TrkB induced the phosphorylation of GSK-3β(Ser9) in all three of these components (Fig. 6A, right).
of the mitochondrial membrane. The BDNF-treated cells had similar scattergram as control. After 8 h of etoposide treatment, the cells in the R4 region increased to 24%, indicating an increase in cells with depolarized mitochondrial membrane. In the cells with pretreatment of BDNF before etoposide, there are 13% of cells in the R4 region (Fig. 6D). This result indicated that BDNF attenuated the mitochondrial membrane potential changes induced by etoposide at 8 h. To study if GSK-3β exerts its role in cell survival via mitochondria, we transfected GSK-3β siRNA into TB8 cells and then treated with etoposide for 24 h. The result showed that there was a 17% decrease in the R4 region cells in GSK-3β siRNA-transfected cells compared with control siRNA-transfected cells after etoposide treatment (Fig. 6E). This means that GSK-3β exerted its role in cell survival via the mitochondria.

Discussion
PI3K/Akt signaling pathway plays a central role in the survival of mammalian cells (30). Our previous studies found that BDNF activation of TrkB activates the PI3K pathway and reduces the sensitivity of neuroblastoma cells to cytotoxic drugs such as etoposide and cisplatin, and constitutively active Akt alone could attenuate the effects of chemotherapy on neuroblastoma cells (9). A variety of substrates of Akt have been implicated in the regulation of cell survival, including Bcl-2 family member Bad (31), caspase 9 (32), the transcription factors Forkhead (33, 34), nuclear factor-κB (35) and protein kinase GSK-3β (36). We focused on GSK-3β because of its known role as a modulator of proapoptotic signals and its involvement in mediating the effects of the Parkinsonian mimic 6-hydroxydopamine on normal neurons and neuroblastoma cells (18).

To evaluate if GSK-3β is involved in cell survival, we inhibited GSK-3β activity using LiCl, pharmacologic GSK-3β inhibitors, and gene silencing with a siRNA targeted to GSK-3β, respectively, or increase the GSK-3β activity by transfecting constitutively active GSK-3β into the neuroblastoma cells before chemotherapy. There was an increase in cell survival after inhibition of GSK-3β and a decrease in cell survival after activation of GSK-3β during the treatment with chemotherapy. The increase in cell survival after GSK-3β silencing by siRNA following chemotherapy is about 13% to 15% and seemed lower than the protection of BDNF and GSK-3β inhibitors. One reason for the relatively low level of protection in these experiments compared with BDNF may be that only 60% to 70% of neuroblastoma cells are transfected, and thus, the 13% to 15% is an underestimation of the potential of the whole population. However, we cannot rule out that other Akt substrates may contribute to the BDNF/TrkB-activated protection of neuroblastoma cells from chemotherapy. In fact, our preliminary evidence indicates that FKHRL1 may be another target that mediates BDNF/TrkB-PI3K-Akt protection of neuroblastoma cells from chemotherapy. Our study supports those that show that GSK-3β functions as a proapoptotic protein (14–19). However, some studies indicate that GSK-3β mediates pro-survival signals (29, 37, 38). At present, the pro- or antiapoptotic effects of GSK-3β are considered to be the result of GSK-3β having opposite effects on the two major apoptotic signaling pathways. GSK-3β promotes signals mediated by the activation of the intrinsic or mitochondria apoptotic signaling pathway, while inhibiting signals mediated by death receptor–induced apoptosis (29). The neuroblastoma cells we used have no detectable level of caspase-8, a requisite effector caspase of death receptor–induced apoptosis, and in our present study, we did not observe...
any antiapoptotic role of GSK-3β. Thus, the primary apoptotic death-inducing pathway would be through the intrinsic or mitochondrial apoptotic pathway. By dynamically measuring the response of the cells to chemotherapy using the RT-CES system, we identified that 6 to 8 h following etoposide treatment was the critical time to determine the cell death or cell survival. Consistent with this, we found that 8 h after etoposide treatment dephosphorylation of GSK-3β and depolarization of mitochondrial membrane were observed in the mitochondria. BDNF pretreatment blocked the etoposide-induced dephosphorylation of GSK-3β and depolarization of mitochondrial membrane in the mitochondria at the key time. In the mitochondrial apoptotic pathway, Bcl-2 family members played a very important role in apoptosis. Maurer (39) reported that GSK-3β promotes the induction of apoptosis by phosphorylation of MCL-1, one of the antiapoptotic proteins in the Bcl-2 family. Other Bcl-2 family members such as Bax have also been reported to be regulated by GSK-3β during apoptosis (40). Our future studies will evaluate the role of Bcl-2 family members in the BDNF/TrkB/PI3K/Akt/GSK-3β protection of neuroblastoma cells from chemotherapy.

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References


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