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Signal Transduction Pathways through TRK-A and TRK-B Receptors in Human Neuroblastoma Cells

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Little is known about the signal transduction pathways of TRK family receptors in neuroblastoma (NB) cells. In this study, an NB cell line, designated MP-N-TS, was established from an adrenal tumor taken from a 2-year-old boy. This cell line expressed both TRK-A and TRK-B receptors, which is rare in a single NB cell line. Therefore, the MP-N-TS cell line was used to determine whether the signal transduction through these constitutive receptors is functional. Three neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), induced tyrosine phosphorylation of panTRK, and BDNF and NT-4/5 induced tyrosine phosphorylation of TRK-B. Tyrosine phosphorylation of panTRK and/or TRK-B by the neurotrophins was inhibited in the presence of a tyrosine kinase inhibitor K252a. Tyrosine phosphorylation of Src homologous and collagen (Shc), extracellular signal-regulated kinase (ERK)-1 and ERK-2, and phospholipase C-γ1 (PLC-γ1) was increased by the three neurotrophins and the increase was inhibited in the presence of K252a. Activation of Ras, detected as the GTP-bound form of Ras, was induced by the three neurotrophins. The neurotrophins did not modulate the expressions of TRK-A or TRK-B mRNA, but they did induce the expression of c-fos mRNA. Exogenous NGF induced weak neurite outgrowth, whereas exogenous BDNF and NT-4/5 induced distinct neurite outgrowth. Exogenous BDNF and NT-4/5 increased the number of viable cells, while NGF did not. Our results demonstrate that the signal transduction pathways through TRK-A and TRK-B in MP-N-TS cells are functional and similar, and the main downstream signaling pathways from the three neurotrophins are mitogen-activated protein kinase (MAPK) cascades through Shc, activated Ras, ERK-1 and ERK-2, and the transduction pathway through PLC-γ1. Further, BDNF and NT-4/5 increased cell viability. The MP-N-TS cell line should be useful for clarifying the TRK-A and TRK-B signaling pathways responsible for the different prognoses in patients with NB.

Key words: Signal transduction — Neuroblastoma — TRK-A — TRK-B — Neurotrophins

Neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), play important roles in the development of the peripheral and central nervous systems. These neurotrophins have several functions in cell survival, cell differentiation, cell proliferation, apoptosis and guidance of the outgrowth of neuronal processes. Neurotrophin function is mediated by the TRK family of tyrosine-kinase receptors (TRK-A, TRK-B and TRK-C).

Neuroblastoma (NB) is one of the common malignant solid tumors in childhood, arising from neural crest precursors. Recently, the clinical heterogeneity of NBs in patients has been revealed to be based on the diverse biological characteristics of these tumor cells. These characteristics include MYCN (N-*myc*) amplification, TRK-A expression and Ha-ras/TRK-A and TRK-B expressions.

Analysis of TRK family receptors in NB cells has shown that NB tumors with a favorable prognosis express high levels of TRK-A, whereas those with an unfavorable prognosis express low levels of TRK-A (or none)[1–4] and high levels of TRK-B.[5,6] NB cell lines established from patients with a poor prognosis sometimes expressed constitutive TRK-A, and seldom expressed constitutive TRK-B.[5,6] In this study, an NB cell line, designated MP-N-TS, was established from a 2-year-8-month-old boy. MP-N-TS constitutively expressed both TRK-A and TRK-B receptors, which is rare for an NB cell line. By using this cell line, we attempted to determine whether these two TRK family receptors are functional, and to clarify the biological and clinical characteristics of the TRK-A and TRK-B signaling pathways in NB cells.

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MATERIALS AND METHODS

Clinical history of the patient The patient was a 2-year-8-month-old Japanese boy who presented with anemia, right-exophthalmus and a small gingival tumor on December 28, 1994. Clinical examination revealed a left upper abdominal mass, and computer-aided tomography of the abdomen showed a primary tumor arising from the left adrenal gland. High levels of the catecholamine metabolites, vanilmandelic acid and homovanillic acid, were identified in the patient's urine. Biopsy of the primary tumor led to the histological diagnosis of stage 4 NB without MYCN amplification. The patient was treated with chemotherapy consisting of a combination of cyclophosphamide, etoposide, doxorubicin and cisplatinum, followed by megatherapy with peripheral blood stem cell transplantation. Although complete remission was achieved, the patient died of brain metastasis in the right temporal lobe on September 7, 1996.

Cell culture A biopsy tumor sample for cell culture was obtained from the primary tumor prior to chemotherapy on January 4, 1995. Cells were cultured in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml) and heat-inactivated fetal calf serum (FCS) (10%) at 37°C in a 5% CO_2 incubator. The medium was changed every 4 to 6 days. Cells were sub-cultured into new flasks by trypsinization when in a sub-confluent state.

Cell lines Thirteen NB cell lines (SMS-KCN, NB-1, GOTO, SMS-KAN, IMR32, KP-N-YN, KP-N-TK, KP-N-YS, MP-N-MS, KP-N-HN, KP-N-RT, SJ-N-CG, and KP-N-SIFA)[7–9] and the rat pheochromocytoma cell line PC12[10] were cultured and used as controls.

Neurotrophins NGF and NT-4/5 were kindly provided by Genentech, Inc. (South San Francisco, CA). BDNF was a gift of AMGEN, Inc. (Thousand Oaks, CA).

Antibodies and reagent The agarose-conjugated anti-phosphotyrosine (pTyr) antibody 4G10 and phosphatidylinositol 3-kinase (PI3-K) (UB93-3) were obtained from UBI (Upstate Biotechnology Inc., Lake Placid, NY). The anti-panTRK (sc-011), anti-extracellular signal-regulated kinase-1 (ERK-1) (sc-93), anti-extracellular signal-regulated kinase-2 (ERK-2) (sc-154) and anti-phospholipase C-γ1 (PLC-γ1) (sc-426) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-TRK-B (p145 and p95), anti-Src homologous and collagen (Shc) antibodies were obtained from Transduction Laboratories (Lexington, KY), and anti-Ras (NC-RAS004) was previously reported.[11] A stock solution of a tyrosine kinase inhibitor K252a (Calbiochem-Novabiochem Corp., La Jolla, CA) was dissolved in dimethyl sulfoxide.[12]

RNA analysis Isolation of total cellular RNA and northern blot analysis were done as described previously.[8] The human TRK-A probe was a 2.7-kb fragment containing all of the coding region.[5] The human TRK-B probe was a 6.0-kb *Eco*RI fragment containing the entire region of full-length TRK-B.[6] The human c-fos probe was a 2.1-kb *Eco*RI fragment of pSPT-fos cDNA.

Immunoprecipitations and immunoblots Cells were seeded onto 100-mm Petri dishes 3 days prior to treatment with neurotrophins. The cells were incubated in serum-free RPMI medium for 2 h on the day of the experiments. For the K252a studies, cells were incubated with a concentration of 200 n*M* K252a for 30 min before the addition of neurotrophins, and were subsequently treated with neurotrophins at 37°C for various times from 0 to 60 min. At the end of incubation, the medium was quickly removed and 150 μl of 1% Nonidet P-40 lysis buffer (20 m*M* Tris pH 8.0, 137 m*M* NaCl, 10% glycerol, 1 m*M* phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 20 μ*M* leupeptin, 1 m*M* sodium vanadate) was added. The cells were scraped from the dishes, transferred to microtubes and sonicated for 1 min. After centrifugation for 20 min at 15 000*g*, the supernatants were used for immunoprecipitation after determination of the protein content (Bio-Rad, Hercules, CA). The volumes used were adjusted so that they each contained the same amount of protein. For the phosphotyrosine immunoprecipitation, the agarose-conjugated pTyr antibody was incubated with the lysates overnight and the immune complexes were collected by centrifugation. Agarose beads precoupled with pTyr antibody were washed 4 times with lysis buffer and the immune complexes were released by addition of 70 μl of Laemmli buffer, then the solution was boiled for 2 min. The immunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) (8%).

The proteins were transferred electrophoretically to nitrocellulose membranes. After blocking of nonspecific binding sites for 1 h at room temperature, the membranes were probed with antibodies. The immunoreactivity was detected after incubation with a peroxidase-labeled anti-species antibody using a non-isotopic chemiluminescent ECL detection system (Amersham, Arlington Heights, IL). The blots were stripped in 62.5 m*M* Tris-HCl pH 6.7 containing 100 m*M* 2-mercaptoethanol and 2% SDS for 30 min at 50°C and reprobed.

Detection of total Ras and GTP-bound form of Ras Total Ras was determined by immunoblotting of total cell lysates with anti-Ras antibody. The activation of Ras was determined as the amount of the GTP-bound form of Ras.[13, 14] Briefly, cDNA of the minimal Ras-binding domain (RBD) of Raf1 (amino acids 51 to 131) was subcloned into the *Bam*HI-*Eco*RI site of pGEX-2T in-frame. Glutathione S-transferase (GST)-RBD fusion protein production was induced in *Escherichia coli* by isopropyl-β-D-thiogalactopyranoside treatment. After sonication, the crude extract was subjected to SDS-PAGE, and GST-RBD fusion protein induction was confirmed by staining with

Coomassie blue. The GTP-bound form of Ras was extracted from the total cellular lysate obtained from cultured cells on Petri dishes with lysis buffer (10 m*M* Tris pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA, 0.1% Triton X100, 5 m*M* $MgCl_2$, 1 m*M* sodium vanadate) by incubation with the GST-RBD protein precoupled with glutathione-Sepharose beads (Pharmacia AB, Uppsala, Sweden) for 1 h at 4°C. The beads were collected by centrifugation, washed with lysis buffer three times, boiled and subjected to SDS-PAGE. Immunoblotting was performed with a murine anti-Ras antibody. Immunoreactive proteins were visualized with an ECL detection system.

Induction of morphological differentiation by neurotrophins Cells were plated onto 60-mm Petri dishes and cultured with RPMI 1640 with 10% FCS. After a 3-day culture period, the medium was replaced with RPMI 1640 with 1% FCS, and neurotrophins were added. The cells were cultured for 7 days, and 200 cells from at least 3 different regions were examined by inverted phase microscopy, and scored as morphologically differentiated, if they possessed one or more processes at least twice as long as the soma diameter.[15]

Cell viability test To quantify the viability of cells, a TetraColor One assay system (Seikagaku Corp., Tokyo) was used. In this assay, a highly water-soluble disulfonated tetrazolium salt, 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate sodium salt (WST-8), was used. This salt, which produces a highly colored formazan dye upon NADH reduction in the presence of 1-methoxy-5-methylphenazium methosulfate (1-methoxy PMS), can be used as a chromogenic reagent for NADH in cell viability or proliferation assay.[16, 17] One hundred microliters of adherent cells (10×10^3 or 20×10^3 cells) in the logarithmic phase was inoculated onto a 96-well microplate in triplicate, and the plate was pre-incubated with RPMI medium with 1% FCS for 24 h in a CO_2 incubator at 37°C. Neurotrophins were added to the wells in triplicate. After 4 days of incubation, a working solution containing WST-8 and 1-methoxy PMS was added to each well and the cells were incubated for an additional 2 h. The absorbance of each well was measured at 450 nm with a reference wavelength of 650 nm, using a microplate reader.

RESULTS

Establishment of the MP-N-TS neuroblastoma cell line Tumor cells obtained from a biopsy sample from the left adrenal tumor grew in the form of an adherent monolayer. The growing spindle-shaped cells have been maintained for more than 70 passages over a 4-year period. This cell line, designated MP-N-TS, was identified as an NB cell line from the clinical features of the patient, and from the increased cellular dopamine and noradrenaline levels, and the result of surface-membrane analysis, as described previously.[7] MYCN was not amplified in MP-N-TS cells.

Expression of TRK-A and TRK-B mRNA in NB cell lines Expression of TRK-A and TRK-B mRNA in the PC12 rat pheochromocytoma cell line, 13 NB cell lines and the MP-N-TS cell line was studied by northern blot analysis (Fig. 1). Of these cell lines, MP-N-TS cells expressed the highest level of TRK-B transcripts. Therefore, in the present study of signal transduction of TRK-A and TRK-B receptors, the MP-N-TS cell line was used.

Dose of neurotrophins inducing tyrosine phosphorylation of panTRK and TRK-B proteins in MP-N-TS cells The optimal doses of neurotrophins for induction of tyrosine phosphorylation of panTRK and TRK-B were determined by the phosphotyrosine immunoprecipitation method. The concentrations of exogenous BDNF of 50, 100 and 200 ng/ml were used, and the maximal tyrosine phosphorylation of panTRK and TRK-B was induced by 100 ng/ml of BDNF and maintained by 200 ng/ml (Fig. 2). Similar experiments indicated that the optimal doses of NGF and NT-4/5 were 100 ng/ml (data not shown). Therefore, for further experiments the doses of 100 ng/ml of neurotrophins were chosen.

Time course of tyrosine phosphorylation of panTRK and TRK-B proteins in MP-N-TS cells The time courses of tyrosine phosphorylation of panTRK and TRK-B were determined by the phosphotyrosine immunoprecipitation method. Exogenous NGF induced high levels of tyrosine phosphorylation of panTRK after 10 min of treatment, although nonspecific higher-molecular-sized bands were observed (Fig. 3). Exogenous BDNF induced high levels of tyrosine phosphorylation of panTRK and full-length TRK-B (145 kD) after 5 min of treatment, although

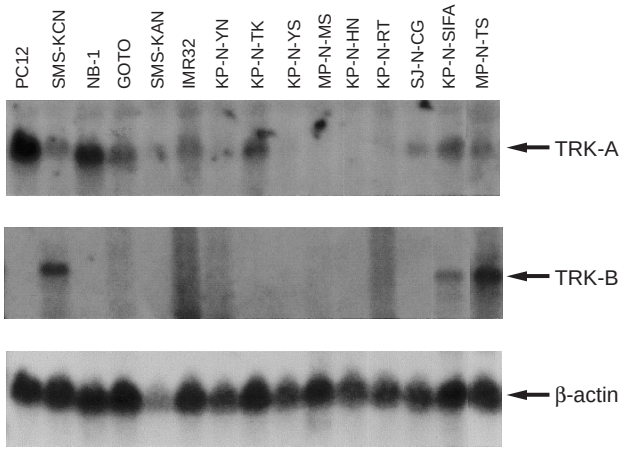

Fig. 1. Northern blot showing expression of TRK-A, TRK-B and β-actin mRNAs in the PC12 pheochromocytoma cell line, a panel of 13 neuroblastoma cell lines, and the MP-N-TS cell line. Expression of β-actin was used as a control.

nonspecific higher-molecular-sized bands were observed as well (Fig. 3). However, it did not induce tyrosine phosphorylation of truncated TRK-B (95 kD) (data not shown). Similar time courses for the tyrosine phosphorylation of panTRK and TRK-B proteins were obtained by treating the cells with NT-4/5 (data not shown). Therefore, as an optimal incubation time for neurotrophins, 10 min was chosen for tyrosine phosphorylation of panTRK, TRK-B and other cellular signaling proteins.

Effect of tyrosine kinase inhibitor K252a on signaling proteins in MP-N-TS cells The neurotrophin treatments increased the tyrosine phosphorylation of panTRK, TRK-B, Shc, ERK-1, ERK-2 and PLC-γ1, while the tyrosine kinase inhibitor, K252a inhibited these neurotrophin-induced changes. The tyrosine phosphorylation of PI3-K was not changed by any of the neurotrophins with or without K252a. Also, low levels of tyrosine phosphorylation of Shc, ERK-1, ERK-2, PLC-γ1 and PI3-K were consistently observed in untreated MP-N-TS cells (Fig. 4).

Induction of GTP-bound form of Ras by neurotrophins MP-N-TS cells were incubated without FCS for 2 h and then stimulated with BDNF (100 ng/ml) for 5 to 60 min. The amount of total Ras (21 kD), determined by immunoblotting from total cell lysates with anti-Ras antibody, was not changed by BDNF treatment for 60 min (Fig. 5, upper). On the other hand, BDNF treatment for 10 min led to the GTP-bound form of Ras (21 kD) (Fig. 5, lower). We then examined the effect of neurotrophins (100 ng/ml) on

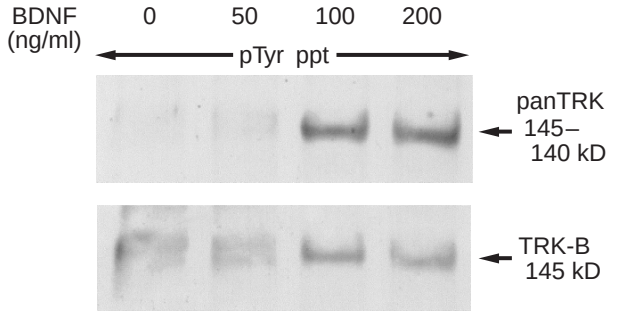

Fig. 2. Dose of BDNF inducing tyrosine phosphorylation of panTRK and TRK-B receptor proteins in MP-N-TS cells. pTyr ppt, precipitated by anti-phosphotyrosine antibody.

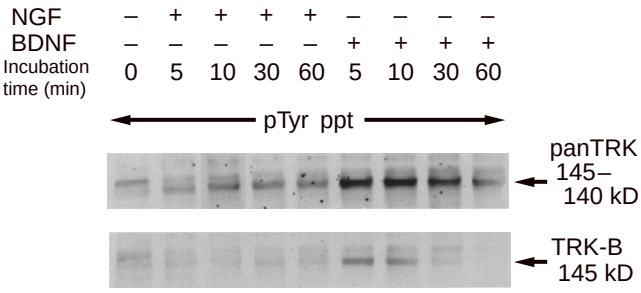

Fig. 3. Time course of the tyrosine phosphorylation of panTRK and TRK-B receptor proteins with NGF and BDNF in MP-N-TS cells. pTyr ppt, precipitated by anti-phosphotyrosine antibody.

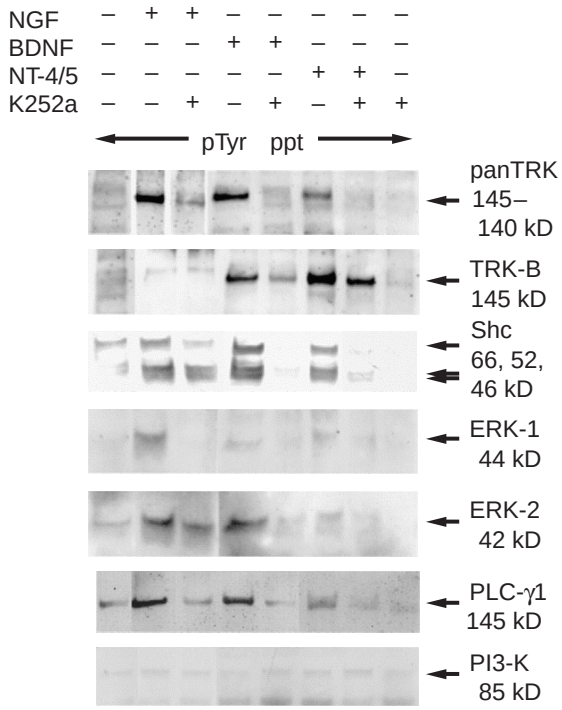

Fig. 4. Effects of tyrosine kinase inhibitor K252a on signaling proteins in MP-N-TS cells in the presence of NGF, BDNF and NT-4/5. pTyr ppt, precipitated by anti-phosphotyrosine antibody.

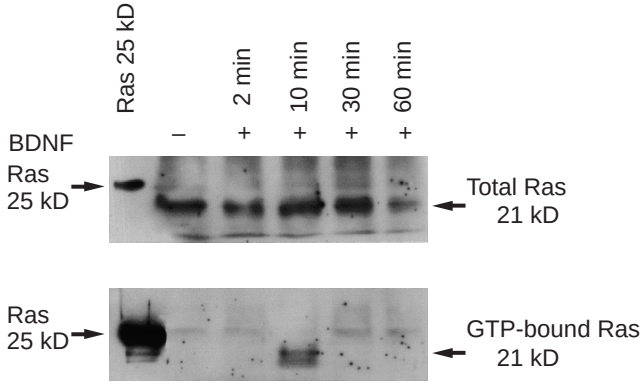

Fig. 5. Induction of the GTP-bound form of Ras with neurotrophins. Time course of the amount of total Ras (21 kD) and the GTP-bound form of Ras (21 kD) with BDNF in MP-N-TS cells.

the GTP-bound form of Ras. The amount of total Ras was not changed by 10 min treatment with NGF, BDNF or NT-4/5 (Fig. 6, upper). However, low levels of the GTP-bound form of Ras were detected after the NGF treatment, and high levels of GTP-bound form of Ras were detected after BDNF and NT-4/5 treatments (Fig. 6, lower). As a positive control for Ras, we used recombinant v-Ha-Ras protein (Figs. 5 and 6, left lanes), which was produced in *E. coli*. This Ras protein has an extra amino acid sequence at its amino-terminus, resulting in an apparent molecular mass of 25 kD.[18)]

Effect of neurotrophins on TRK-A, TRK-B and c-fos mRNA expressions MP-N-TS cells were treated with neurotrophins (100 ng/ml) for 10 min. The expressions of TRK-A and TRK-B mRNA were not changed. However, the expression of c-fos mRNA, an immediate-early gene, was faintly induced by NGF, and clearly induced by BDNF and NT-4/5 (Fig. 7).

Induction of morphological differentiation by neurotrophins in MP-N-TS cells In the absence of neurotrophins, MP-N-TS cells spontaneously extended short neurites and 10% of cells appeared differentiated after culture for 7 days (Fig. 8A). In the presence of exogenous NGF (100 ng/ml), a weak enhancement of neurite outgrowth was observed and 32% of cells appeared differentiated (Fig. 8B). However, the addition of exogenous BDNF (100 ng/ml) and NT-4/5 (100 ng/ml) clearly enhanced neurite outgrowth and 73% and 75% of cells were differentiated, respectively (Fig. 8, C and D).

Effects of neurotrophins on cell viability The effect of treatment with neurotrophins (100 ng/ml) for 4 days on the viability of MP-N-TS cells was studied. Five separate experiments were performed, and the results of each experiment were similar. The results of one of those experiments are shown in Fig. 9. NGF had no significant

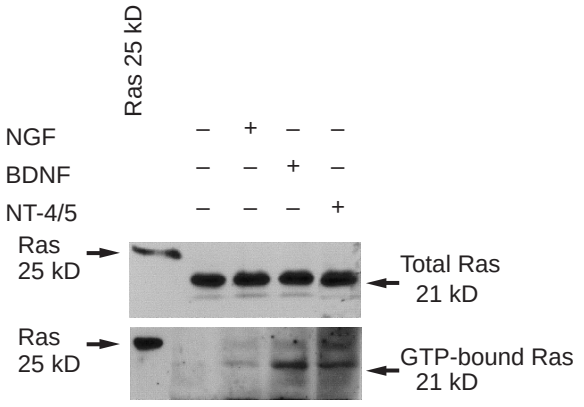

Fig. 6. Induction of the GTP-bound form of Ras with neurotrophins. Effect of NGF, BDNF and NT-4/5 on the amount of total Ras (21 kD) and the GTP-bound form of Ras (21 kD) in MP-N-TS cells.

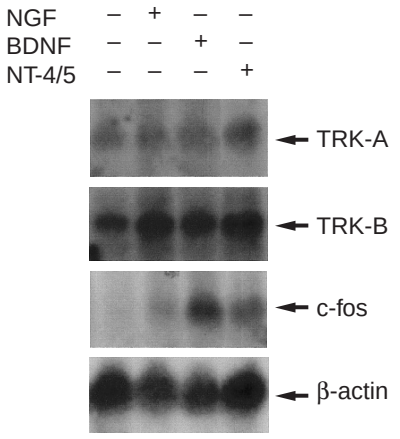

Fig. 7. Effects of NGF, BDNF and NT-4/5 on TRK-A, TRK-B, c-fos and β-actin mRNAs expressions in MP-N-TS cells. Expression of β-actin was used as a control.

effect on cell viability as compared with the control culture, whereas BDNF and NT-4/5 significantly increased the cell numbers during the 4-day culture period (151% and 157% enhancements at 20×10^3 cells/well, respectively) (Student's *t* test; $P<0.01$).

DISCUSSION

There is accumulating evidence that tyrosine protein kinase receptors of the TRK family (TRK-A, TRK-B and TRK-C) serve as signaling receptors for the neurotrophins (NGF, BDNF, NT-3 and NT-4/5). TRK-A is the signaling receptor for NGF, TRK-B is the signaling receptor for BDNF and NT-4/5, and TRK-C is the primary receptor of NT-3. However, TRK-A and TRK-B can be activated in part by NT-3, at least in fibroblasts or neuronal cells.[19–23)]

Among these TRK family receptors, the relationship between signal transduction of NGF and TRK-A has been extensively studied with the PC12 rat pheochromocytoma cell line.[22, 24)] In addition, the signal transduction of neurotrophins has been investigated in constitutive TRK receptors of cultured primary neurons[25, 26)] and in TRK-transfected neuronal or fibroblast cells.[19, 22)] However, few studies on the signal transduction of neurotrophins in human NB cell lines have been undertaken, since the dual expression of *TRK-A* and *TRK-B* genes in a single NB cell line is rare.[5, 27)]

Several studies have demonstrated that a high expression of TRK-A in NB tumors is correlated with good prognosis, whereas negative and low expressions of TRK-A in NB tumors have been correlated with poor prognosis.[1–4)] However, we recently analyzed the expression of TRK-A in our panel of 18 NB cell lines, which were derived from patients with poor prognosis. The *TRK-*

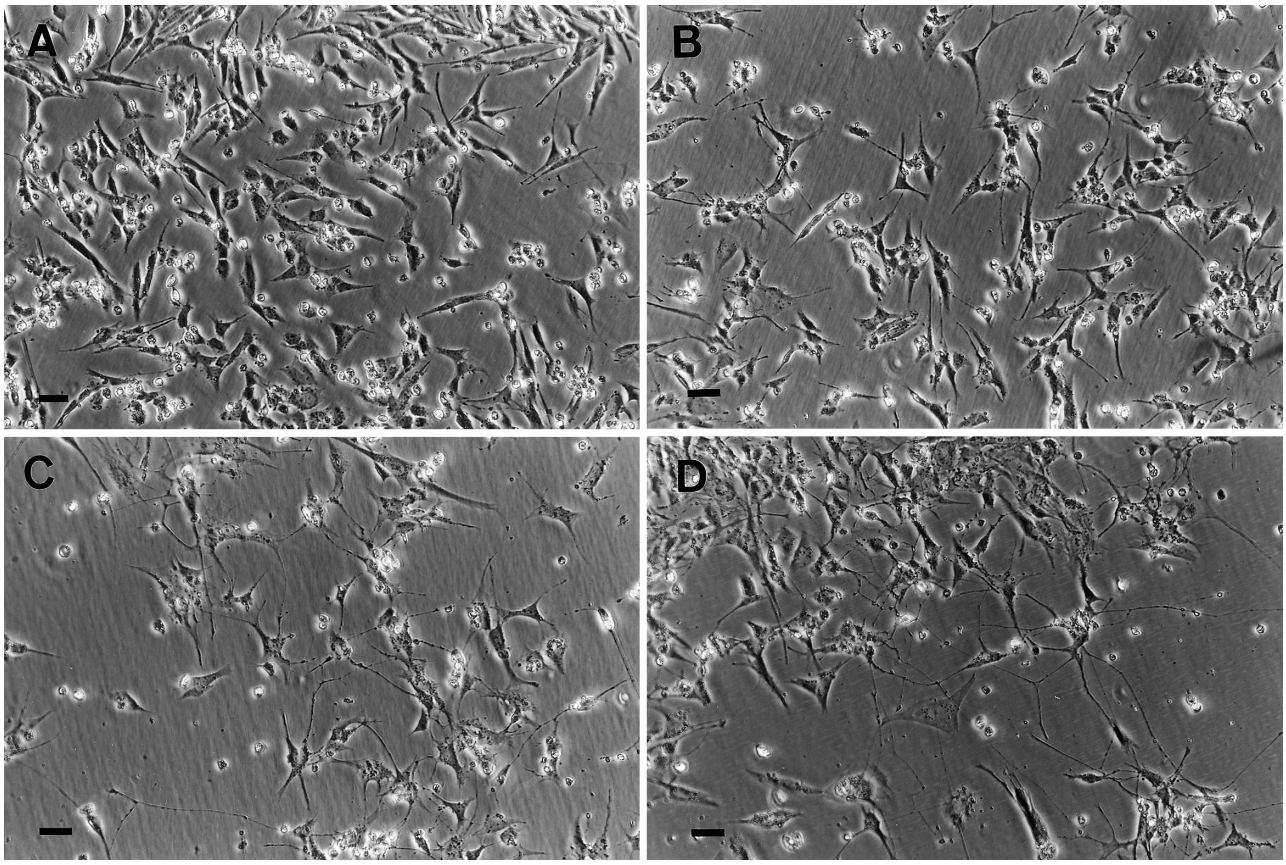

Fig. 8. Induction of morphological differentiation by neurotrophins in MP-N-TS cells. Control (A), NGF-treated cells (B), BDNF-treated cells (C) and NT-4/5-treated cells (D) (×200). Scale bars=25 μm.

A gene was expressed in seven (39%) of these NB cell lines as detected by northern blotting, and in 11 (61%) of them by RT-PCR, indicating that many NB cell lines express TRK-A.[28] These results prompted us to examine whether signal transduction through TRK-A in NB cell lines is functional.

Expression of the *TRK-B* gene in NB tumors has been reported to be associated with a poor prognosis.[5, 6] However, only one of the 11 NB cell lines examined, SMS-KCN, was found to constitutively express the *TRK-B* gene.[5, 6] Therefore, to clarify signal transduction through TRK-B in NB cells, TRK-B-transfected NB cell lines,[27, 29] or retinoid-treated NB cell lines have been utilized to induce TRK-B expression.[29–33] However, the transfected-*TRK-B* gene does not necessarily function in the same way as the constitutive *TRK-B* gene, since the signal transduction pathway downstream of the TRK-B receptor might be blocked. Therefore, an NB cell line that expresses both *TRK-A* and *TRK-B* genes would be valuable for clarifying the signal transduction pathways of neurotrophins in NB cells.

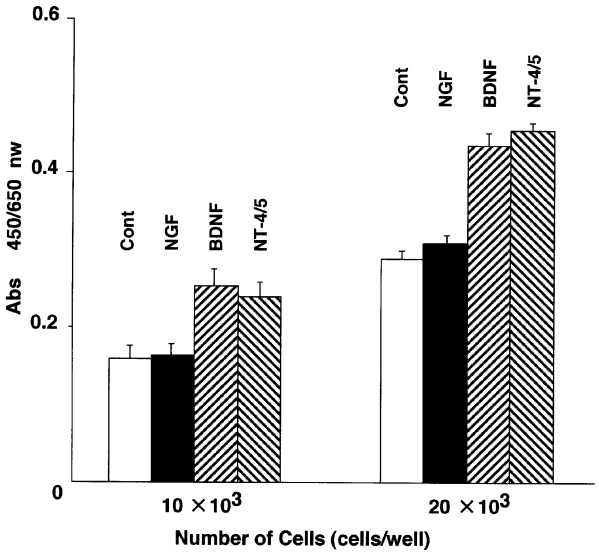

Fig. 9. Effects of NGF, BDNF and NT-4/5 on cell viability in MP-N-TS cells. Values are shown as mean±standard deviations in triplicate cultures.

In the present study, first, the expressions of TRK-A and TRK-B were analyzed by northern blotting in our panel of 14 NB cell lines. Three NB cell lines (SMS-KCN, KP-N-SIFA and MP-N-TS) were found to express both the *TRK-A* and *TRK-B* genes, and of these three cell lines, the MP-N-TS cell line was used in this study, because it showed the highest TRK-B expression (Fig. 1).

The initial step of signal transduction by neurotrophins was the tyrosine phosphorylation of TRK family receptors, followed by the activation of cellular signal proteins. The proteins that signal through TRK family receptors have been extensively studied in PC12 cells and primary neuronal cells. These studies demonstrated that Shc, activation of Ras, MAP kinase including ERK-1 and ERK-2, PLC-γ1 and PI3-K play major roles in signal transduction.[21, 22]

In NB cells, the regulation of TRK-A and TRK-B receptors themselves by retinoic acid and/or γ-interferon has been analyzed,[22, 31, 33, 34] though the signal transduction pathway downstream of TRK family receptors has not been well-characterized. Only one study on the signal transduction pathway downstream of TRK-A and TRK-B receptors in NB cells has been reported.[5]

In MP-N-TS cells, examination of the effects of the three neurotrophins on tyrosine phosphorylation of panTRK and/or full-length TRK-B showed that the optimal incubation time with neurotrophins is 10 min (Fig. 2) and the optimal dose of neurotrophins is 100 ng/ml (Fig. 3). NGF induced the tyrosine phosphorylation of panTRK, but not that of TRK-B receptor proteins, and the NGF-induced tyrosine phosphorylation of panTRK protein was diminished by K252a, an inhibitor of TRK kinase. With regard to the signal receptor for NGF, three anti-TRK-A antibodies used in our preliminary study, which were obtained from different sources, did not specifically recognize TRK-A (data not shown). However, the signal receptor for NGF is known to be TRK-A alone,[21] so in our study, the signal transduction of NGF must be mediated through the tyrosine phosphorylation of TRK-A receptor. In contrast, BDNF and NT-4/5 induced the tyrosine phosphorylation of panTRK and full-length TRK-B receptor proteins, and this tyrosine phosphorylation of the receptor proteins was diminished by K252a, indicating that the signal transductions of BDNF and NT-4/5 are mediated through TRK-B receptor (Fig. 4).

The increased tyrosine phosphorylation of cellular signal proteins with neurotrophins and the blocking effect of K252a revealed that the signal transductions downstream of the three neurotrophins include both MAP kinase cascades through Shc, ERK-1 and ERK-2, and the signaling pathway through PLC-γ1. However, the signaling pathway through PI3-K is not likely to work in MP-N-TS cells. Low levels of tyrosine phosphorylation of Shc, ERK-1, ERK-2, PLC-γ1 and PI3-K were observed in untreated MP-N-TS cells (Fig. 4). One explanation for the native phosphorylation of these signal proteins is that BDNF but not NGF, detected in MP-N-TS cells by RT-PCR (data not shown), may act on MP-N-TS cells in an autocrine manner, or alternatively, the starvation period for 2 h before neurotrophin treatment may not be enough to eliminate the native phosphorylation.

The appearance of the GTP-bound form of Ras in the presence of the three neurotrophins demonstrated that the activation of Ras is one of the major pathways of action of neurotrophins in MP-N-TS cells (Figs. 5 and 6). This signal cascade is similar to the TRK-A signal transduc-tion pathway of NGF in PC12 rat pheochromocytoma cells.[10, 22]

The expressions of TRK-A and TRK-B mRNA were not modulated by treatment with any of the neurotrophins. However, the expression of c-fos, one of the immediate-early genes, was induced weakly by NGF, but distinctly by BDNF and NT-4/5, indicating that the signals of neurotrophins are finally transduced to the nucleus in MP-N-TS cells (Fig. 7).

A morphological differentiation in terms of neurite outgrowth was induced by the three neurotrophins (Fig. 8). Exogenous BDNF and NT-4/5 increased the numbers of viable cells, while NGF had no significant effect on cell numbers (Fig. 9). To clarify how BDNF and NT-4/5 increased the numbers of viable cells, cell cycle analysis was conducted and the phosphorylation state of retinoblastoma protein[35] was determined at various stages of the cell cycle. However, no effect of BDNF and NT-4/5 was found (data not shown). Therefore, the increase in the number of viable cells by BDNF and NT-4/5 is probably caused by increased cell survival rather than by cell proliferation.

The association of aggressive tumor phenotype, high metastatic ability and chemoresistant phenotypes with TRK-B expression in NB cells has been reported.[5, 6, 29, 32, 33] There may be a relationship between the autocrine production system of BDNF and the signal pathway through TRK-B in NB cells, and this relationship may be partly responsible for the aggressive characteristics of NB cells.

An NB cell line expressing constitutive TRK-A and TRK-B receptors is rare. Our results demonstrate that the signal transduction pathways through TRK-A and TRK-B receptors in MP-N-TS cells are functional and similar. However, BDNF and NT-4/5 increased cell viability, whereas NGF did not. This MP-N-TS cell line could be useful for clarifying the diverse TRK family receptor-mediated signaling pathways, including those leading to neuronal differentiation, proliferation, survival, apoptosis and drug resistance in NB cells.

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REFERENCES

1) Nakagawara, A., Nakagawara-Arima, M., Scarvarda, N. J., Azar, C. G., Cantor, A. B. and Brodeur, G. M. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N. Engl. J. Med.*, **328**, 847–854 (1993).
2) Suzuki, T., Bogenmann, E., Shimada, H., Stram, D. and Seeger, R. Lack of high-affinity nerve growth factor receptors in aggressive neuroblastoma. *J. Natl. Cancer Inst.*, **85**, 377–384 (1993).
3) Kogner, P., Barbany, G., Dominici, C., Castello, M. A., Raschella, G. and Persson, H. Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. *Cancer Res.*, **53**, 2044–2050 (1993).
4) Tanaka, T., Sugimoto, T. and Sawada, T. Prognostic discrimination among neuroblastomas according to Ha-ras/trk A gene expression. *Cancer*, **83**, 1626–1633 (1998).
5) Nakagawara, A., Azar, C. G., Scavarda, N. J. and Brodeur, G. M. Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol. Cell. Biol.*, **14**, 759–767 (1994).
6) Nakagawara, A., Liu, X. G., Ikegaki, N., White, P. S., Yamashiro, D. J., Nycum, L. M., Biegel, J. A. and Brodeur, G. M. Cloning and chromosomal localization of the human TRK-B tyrosine kinase receptor gene (NTRK2). *Genomics*, **25**, 538–546 (1995).
7) Sugimoto, T., Sawada, T., Matsumura, T., Kemshead, J. T., Ishii, T., Horii, Y., Morioka, H., Morita, M. and Reynolds, C. P. Identical expression of cell surface membrane antigens on two parent and eighteen cloned cell lines derived from two different neuroblastoma metastases of the same patient. *Cancer Res.*, **46**, 4765–4769 (1986).
8) Sugimoto, T., Ueyama, H., Hosoi, H., Inazawa, J., Kato, T., Kemshead, J. T., Reynolds, C. P., Gown, A. M., Mine, H. and Sawada, T. Alpha-smooth-muscle actin and desmin expressions in human neuroblastoma cell lines. *Int. J. Cancer*, **48**, 277–283 (1991).
9) Matsumura, T., Sugimoto, T., Sawada, T., Amagai, T., Negoro, S. and Kemshead, J. T. Cell surface membrane antigen present on neuroblastoma cells but not fetal neuroblasts recognized by a monoclonal antibody (KP-NAC8). *Cancer Res.*, **47**, 2924–2930 (1987).
10) Muroya, K., Hattori, S. and Nakamura, S. Nerve growth factor induces rapid accumulation of the GTP-bound form of p21ras in rat pheochromocytoma PC12 cells. *Oncogene*, **7**, 277–281 (1992).
11) Kanai, T., Hirohashi, S., Noguchi, M., Shimoyama, Y., Shimosato, Y., Noguchi, S., Nishimura, S. and Abe, O. Monoclonal antibody highly sensitive for the detection of *ras* p21 in immunoblotting analysis. *Jpn. J. Cancer Res.* (Gann), **78**, 1314–1318 (1987).
12) Tapley, P., Lamballe, F. and Barbacid, M. K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. *Oncogene*, **7**, 371–381 (1992).
13) de Rooji, J. and Bos, J. L. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene*, **14**, 623–625 (1997).
14) Matsumura, I., Nakajima, K., Wakao, H., Hattori, S., Hashimoto, K., Sugahara, H., Kato, T., Miyazaki, H., Hirano, T. and Kanakura, Y. Involvement of prolonged Ras activation in thrombopoietin-induced megakaryocytic differentiation of a human factor dependent hematopoietic cell line. *Mol. Cell. Biol.*, **18**, 4282–4290 (1998).
15) Sugimoto, T., Sawada, T., Matsumura, T., Horii, Y., Kemshead, J. T., Suzuki, Y., Okada, M., Tagaya, O. and Hino, T. Morphological differentiation of human neuroblastoma cell lines by a new synthetic polyprenoic acid (E5166). *Cancer Res.*, **47**, 5433–5438 (1987).
16) Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y. and Ueno, K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta*, **44**, 1299–1305 (1997).
17) Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K. and Watanabe, M. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Anal. Commun.*, **36**, 47–50 (1999).
18) Lautenberger, J. A., Ulsh, L., Shih, T. Y. and Papas, T. S. High-level expression in *Escherichia coli* of enzymatically active Harvey murine sarcoma virus p21ras protein. *Science*, **221**, 858–860 (1983).
19) Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., Burton, L. E., Stanton, B. R., Kaplan, D. R., Hunter, T., Nikolics, K. and Parada, L. F. The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell*, **65**, 895–903 (1991).
20) Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Frandl, J., Greene, L. A., Barbacid, M. and Yancopoulos, G. D. Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. *Neuron*, **10**, 137–149 (1993).
21) Barbacid, M. The Trk family of neurotrophin receptors. *J.*

Neurobiol., **25**, 1386–1403 (1994).
22) Kaplan, D. R. and Stephens, R. M. Neurotrophin signal transduction by the Trk receptor. *J. Neurobiol.*, **25**, 1404–1417 (1994).
23) Davies, A. M., Minichiello, L. and Klein, R. Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. *EMBO J.*, **14**, 4482–4489 (1995).
24) Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A. and Kaplan, D. R. Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. *Neuron*, **12**, 691–705 (1994).
25) Zirrgiebel, U. and Lindholm, D. Constitutive phosphorylation of TrkC receptors in cultured cerebellar granule neurons might be responsible for the inability of NT-3 to increase neuronal survival and to activate p21 Ras. *Neurochem. Res.*, **21**, 851–859 (1996).
26) Yamada, M., Ohnishi, H., Sano, S., Nakatani, A., Ikeuchi, T. and Hatanaka, H. Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. *J. Biol. Chem.*, **272**, 30334–30339 (1997).
27) Lucarelli, E., Kaplan, D. and Thiele, C. J. Activation of trk-A but not trk-B signal transduction pathway inhibits growth of neuroblastoma cells. *Eur. J. Cancer*, **33**, 2068–2070 (1997).
28) Horii, Y., Kuroda, H. and Sugimoto, T. Frequent detection of TrkA expression in human neuroblastoma cell lines. *Acta Paediatr. Jpn.*, **40**, 644–646 (1998).
29) Scala, S., Wosikowski, K., Giannakakou, P., Valle, P., Biedler, J. L., Spengler, B. A., Lucarelli, E., Bates, S. E. and Thiele, C. J. Brain-derived neurotrophic factor protects neuroblastoma cells from vinblastine toxicity. *Cancer Res.*, **56**, 3737–3742 (1996).
30) Kaplan, D. R., Matsumoto, K., Lucarelli, E. and Thiele, C. J. Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. *Neuron*, **11**, 321–331 (1993).
31) Lucarelli, E., Kaplan, D. R. and Thiele, C. J. Selective regulation of TrkA and TrkB receptors by retinoic acid and interferon-γ in human neuroblastoma cell lines. *J. Biol. Chem.*, **270**, 24725–24731 (1995).
32) Matsumoto, K., Wada, R. K., Yamashiro, J. M., Kaplan, D. R. and Thiele, C. J. Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cells. *Cancer Res.*, **55**, 1798–1806 (1995).
33) Middlemas, D. S., Kihl, B. K., Zhou, J. and Zhu, X. Brain-derived neurotrophic factor promotes survival and chemoprotection of human neuroblastoma cells. *J. Biol. Chem.*, **274**, 16451–16460 (1999).
34) Shikata, A., Sugimoto, T., Hosoi, H., Sotozono, Y., Shikata, T., Sawada, T. and Parada, L. F. Increased expression of *trk* proto-oncogene by γ-interferon in human neuroblastoma cell lines. *Jpn. J. Cancer Res.*, **85**, 122–126 (1994).
35) Li, H., Kawasaki, H., Nishida, E., Hattori, S. and Nakamura, S. Ras-regulated hypophosphorylation of the retinoblastoma protein mediates neuronal differentiation in PC12 cells. *J. Neurochem.*, **66**, 2287–2294 (1996).