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Original Paper

Role of Neurotrophins and Their Receptors in Human Neuroblastomas: a Primary Culture Study

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Expression of *trk* family genes are prognostic indicators of neuroblastoma. However, the functional role of neurotrophins and their receptors in neuroblastomas *in vivo* is still unclear. We studied the expression of neurotrophin receptors (trk-A, trk-B, trk-C) and their responsiveness to neurotrophins (NGF, BDNF, NT-3) in 25 human neuroblastomas using a primary culture system. The tumours in early stages and stage 4s responded to both NGF and NT-3, but not to BDNF, by surviving and differentiating terminally and the responsiveness was correlated with high levels of trk-A, especially the neuronal isoform. However, in many advanced stage tumours, the expression of trk-A was down-regulated and the response pattern to neurotrophins was diverse, without showing terminal differentiation. Interestingly, a stage 4 tumour with *MYCN* amplification which expressed high level of neuronal trk-A was dependent on nerve growth factor (NGF) for both survival and differentiation in primary culture. The results suggest that the NGF/trk-A signalling may be the main regulatory pathway for differentiation and survival of neuroblastoma *in vivo* and that trk-A overexpression may overcome aggressiveness, even of the tumour with *MYCN* amplification. © 1997 Elsevier Science Ltd.

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INTRODUCTION

NEUROBLASTOMA IS a paediatric tumour originating from sympathoadrenal progenitors derived from the neural crest [1]. Recent studies have revealed that growth, differentiation and survival of neural crest-derived cells are strongly regulated by neurotrophins and their receptors [2–5].

The nerve growth factor (NGF) was the first neurotrophin to be identified [6] and it is the prototype of a family of homologous neurotrophins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 or -5 (NT-4) [2–5]. Recently, the corresponding high-affinity neurotrophin receptor genes have been identified and they have all been found to encode receptor tyrosine kinases. Analysis of the ligand–receptor interactions has shown that trk-A is a receptor for NGF and NT-3, trk-B is a receptor for BDNF as well as NT-4 and NT-3 and trk-C is a receptor for NT-3. $p75^{LNTR}$, which was originally cloned as a low-affinity NGF receptor, binds to all neurotrophins similarly [2–5].

Recent investigations have disclosed that trk-A mRNA is highly expressed in favourable neuroblastomas [7–11], while trk-B mRNA is preferentially expressed in advanced stage, aggressive neuroblastomas with *MYCN* amplification [13]. However, the functional roles of neurotrophins and their receptors in human neuroblastomas *in vivo* is still unclear.

We studied 25 primary neuroblastomas in short-term culture to see the effect of NGF, BDNF and NT-3 on tumour cell survival and differentiation. Our data suggest that in favourable neuroblastomas expressing high levels of trk-A, NGF appears to be an important regulator of differentiation and survival of the tumour cells *in vivo*. In contrast, in the unfavourable neuroblastomas, neurotrophin signalling seems to be defective and expression of trk-A (but not trk-B) is down-regulated, correlating with the lack of responsiveness to NGF.

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PATIENTS AND METHODS

Patients and tumour samples

Patient 1 was diagnosed in the Iowa University Hospital, Iowa, Ohio, U.S.A., in September, 1991. Patients 2–12 were diagnosed between October, 1991 and July 1993, at the Saint Louis Children's Hospital, St. Louis, Missouri, U.S.A. Patients 13–25 were diagnosed between August, 1993 and March, 1995, at the Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, U.S.A. All tumour samples were obtained from surgical biopsies, except for case 14, where tumour cells were obtained by bone marrow aspiration. All tumour samples except two (cases 21 and 23) were obtained before starting chemotherapy or radiation therapy.

Primary culture of neuroblastomas

Fresh tumour tissue (0.2–1.0 g) was cut into very small pieces and incubated in standard culture medium containing 500 units/ml of collagenase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) for 1 h at 37°C at 5% CO_2–air in a 100×20 mm polystyrene dish (Becton Dickinson & Company, Lincoln Park, New Jersey, U.S.A.). Afterwards, tumour cells were dispersed using a plastic syringe with an 18-gauge needle and then the cells were filtered through a metal sieve (40 mesh screen, Sigma Chemical Co.). The cell suspension was centrifuged at 250*g* for 5 min, resuspended in 5–10 ml of standard culture medium, layered on top of 5 ml of lymphocyte separation medium (Organon Teknika Co., Durhan, North Carolina, U.S.A.) and centrifuged at 1000*g* for 10 min. The cells at the interface were removed and washed once or twice with the standard culture medium. The viable, nucleated cells were counted after staining with trypan blue (Sigma Chemical Co.). In order to evaluate the effects of neurotrophins on primary neuroblastoma cells, 2–5 (usually 5)×10^5 cells in 1 ml standard medium were plated per well (precoated with collagen) in 24-well Falcon tissue culture plates (Becton Dickinson & Company). The cells were incubated in 5% CO_2–air at 37°C, and the medium was changed every 3 to 4 days.

Northern blot analysis

Procedures for isolating total cellular RNA and blot hybridisation have been described previously [8]. 25 μg of each RNA were resolved on 1% agarose–formaldehyde gels. trk-A mRNA expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as arbitrary density units (du).

Reverse transcriptase–polymerase chain reaction (RT–PCR)

The annealing reaction was performed at 68°C for 5 min in a 9 μl reaction mixture containing 1 μg of total RNA (or 50 ng of polyA$^+$ RNA) and 300 ng of oligo-d$(T)_{15}$ primer (Promega Corp., Madison, Wisconsin, U.S.A.) and/or 30 pmol of each specific primer, then chilled on ice. The mixture was supplemented with 4 μl of 5 X RT buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine, 50 mM dithiothreitol), 2 μl of 100 μg/ml bovine serum albumin (BSA), 2 μl of 2.5 mM each of dNTPs, 20 units/μl of RNasin (Promega Corp.) and 12.5 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corp.) to a volume of 20 μl, then incubated at 42°C for 2 h. The RT reaction product was diluted with 80 μl of water, then boiled for 5 min, and chilled on ice.

PCR reactions were performed in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 mM NH_4Cl, 100 μM each of dNTPs, 0.5 units of Perfect Match (Stratagene Cloning Systems, La Jolla, California, U.S.A.), 2 μM each 5′ and 3′ PCR primers, 0.1 unit Taq DNA polymerase (Perkin-Elmer Cetus, Branchburg, New Jersey, U.S.A.) and 1 μl of the RT reaction product in a volume of 5 μl. The amplification was performed for 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and the final extension was at 72°C for 7 min. The PCR products were run on 6% Visigel (Stratagene Cloning Systems).

RESULTS

Twenty-five fresh neuroblastoma tissues were processed directly for the primary culture study *in vitro*. However, 4 tumours with histological differentiation and 4 other tumours which had received chemotherapy and/or radiation therapy were excluded from the present analysis because they died within 1 or 2 days of culture, without responding to any neurotrophins.

Of 12 tumours without *MYC*N amplification, 9 (4 stage 1, 2 stage 2, 1 stage 4S and 2 stage 3) showed a consistent pattern of response to neurotrophins (Table 1). Exogenous NGF at a concentration of 100 ng/ml induced extensive neurite outgrowth from tumour cell clumps after a few days of culture and the neuritic processes became thick and straight in time, connecting with the neighbouring tumour cells, tumour cell clusters or fibroblast-like cells. However, the majority of tumour cells cultured in standard medium without adding any exogenous factors started to die within 1 or 2 days, and most cells died within 7–10 days of culture. Three tumours without *MYC*N amplification did not respond to NGF (Table 1), two of which (case 16 and case 19) were early stage tumours and had a favourable histology based on the Shimada classification.

NT-3 at a concentration of 100 ng/ml also promoted survival and induced differentiation of tumours without *MYC*N amplification (Table 1). However, the effects of NT-3 were observed only in tumour cells that were also responsive to NGF, and the effects were consistently weaker than those of NGF at concentrations ranging from 1 to 100 ng/ml. In contrast to NGF and NT-3, BDNF showed no significant effect on this group of tumour cells, even if expression of full-length TRK-B, a cognate receptor of BDNF, was detectable by Northern blot hybridisation (cases 10 and 18 in Table 1).

Four of five stage 4 neuroblastomas with *MYC*N amplification did not show NGF-induced terminal differentiation (Table 1). However, the primary culture cells obtained from both cases 9 and 15 showed some spontaneous neurite extension, and they responded to all neurotrophins tested by extending more neutires, but without ceasing cell proliferation. Both tumours eventually became cell lines.

Surprisingly, the tumour cells obtained from case 8 responded to both NGF and NT-3 (but not to BDNF) by undergoing terminal differentiation, even though the *MYC*N oncogene was amplified in this tumour. Furthermore, in the absence of exogenous NGF or NT-3, the cells died.

The differentiation of primary neuroblastoma cells induced by NGF or NT-3 was well correlated with high levels of *trk*-A expression, especially the neuronal form (Table 1).

The expression of neurotrophins was measured in tumour tissues by RT–PCR (data not shown). The NGF message was detectable in almost all primary tumours. Both BDNF and NT-4, which are common ligands for the trk-B receptor, were also expressed in all primary neuroblastomas and

Table 1. *Relationship between the response to neurotrophins in human neuroblastoma primary culture cells and the expression of neurotrophin receptors in primary neuroblastomas*

Case no.	Age (mo)/ stage	Origin	*MYCN* (copies)	Response to			mRNA expression			
				NGF	BDNF	NT-3	trk-A (du)	trk-B	trk-C	LNTR
Neuroblastomas without *MYCN* amplilication										
13	1/1	Adrenal	1	++	–	+	250 (II)	–	–	+
3	2/1	Pelvis	1	++	n.d.	n.d.	280 (II)	–	–	+
5	14/1	Chest	1	++	–	+	550 (II)	–	+	–
16	15/1	Chest	1	–	±	±	n.d.	n.d.	n.d.	n.d.
11	18/1	Adrenal	1	++	±	+	300 (II)	–	–	±
10	0/2	Adrenal	1	++	–	+	270 (II)	+	+	+
19	8/2	Neck	1	–	–	–	n.d.	n.d.	n.d.	n.d.
22	13/2	Chest	1	++	–	+	530 (II)	–	–	+
14	1/4S	Adrenal	1	++	–	+	n.d.	n.d.	n.d.	n.d.
2	15/3	Chest	1	++	n.d.	n.d.	190 (II)	–	–	+
18	36/3	Abdominal symptoms	1	++	–	+	370 (II)	+	–	+
12	108/4	Abdomen	1	–	–	–	(–)	(–)	(–)	(–)
Neuroblastomas with *MYCN* amplilication										
9	19/4	Adrenal	150	[+]	[+]	[+]	30 (I = II)	+	+	–
15	23/4	Adrenal	150	[+]	[+]	[+]	95 (I = II)	+	+	–
8	31/4	Adrenal	125	++	–	+	320 (II)	–	–	–
21	33/4	Adrenal	>10	–	–	–	n.d.	n.d.	n.d.	n.d.
24	35/4	Abdomen	60	–	–	–	5 (II)	–	–	–

n.d.: not done. The relative responsiveness to neurotrophins are shown, based on cell survival, the number of neurites and the length and thickness of neurites extended, after subtracting the findings of the control. ++, + and – shows the relative grade of terminal differentiation induced, among the same primary culture cells. ± means only modest neurites outgrowth over background. [+]: only neurite extension was induced without cessation of proliferation. The expression of *trk*-A is shown as arbitrary density units (du). (II): predominant expression of the neuronal trk-A_{II} isoform, based on the results of RT–PCR. (I = II): equivalent expression of both non-neuronal trk-A_I isoform and neuronal trk-A_{II} isoform. The expression of trk-B (full-length), trk-C (full-length) and LNTR is based on their detection by Northern blot hybridisation. (–), in case 12 mRNA expression was not detectable by the RT–PCR procedure.

neuroblastoma cell lines. In contrast, the NT-3 message was not detectable in any primary neuroblastomas.

DISCUSSION

Since tumour cell lines do not necessarily reflect the original characteristics of primary tumours, a primary tumour cell culture system may be the best way to evaluate the function of biological pathways of neuroblastomas *in vivo*. The present study demonstrated at least three distinct patterns of neurotrophin responsiveness in different subsets of human neuroblastomas in primary culture. Most favourable neuroblastomas responded to both NGF and NT-3 by surviving and differentiating terminally and this responsiveness was correlated with the high levels of *trk*-A expression. A similar response pattern was also observed in one stage 4 neuroblastoma with *MYCN* amplification but also with high levels of *trk*-A expression. This patient is alive without disease at 31 months after diagnosis. Two stage 4 tumours with *MYCN* amplification expressed intermediate levels of *trk*-A and detectable levels of *trk*-B and *trk*-C by Northern blot analysis. The tumour cells responded to NGF, BDNF and NT-3 by the extension of neurites, but they continued to grow. Finally, three stage 4 neuroblastomas showed no response to any neurotrophins tested and such tumours expressed very low or undetectable levels of the neurotrophin receptors.

Neuroblastomas are usually encapsulated and the tumour tissue can be isolated from the surrounding tissues, except for intrinsic blood vessels. From RT–PCR analysis, NGF, BDNF and NT-4 (but not NT-3) were expressed in tumour tissues (A. Nakagawara, unpublished data), suggesting that NT-3 signalling through any trk receptor can only occur from an exogenous ligand *in vivo*. Therefore, NGF signalling through the neuronal isoform of trk-A seems to be a principal regulator of differentiation and survival for most favourable neuroblastomas and apoptotic tumour cell death may be induced in its absence.

The role of BDNF (NT-4)/trk-B signalling is also important in more aggressive neuroblastomas [13, 14]. In a previous report, we found that full-length *trk*-B is preferentially expressed in neuroblastomas with *MYCN* amplification [13]. In the SMS-KCN neuroblastoma cell line, which endogenously expresses both full-length and truncated *trk*-B, exogenous BDNF induces tyrosine phosphorylation of *trk*-B, phosphorylation of intracellular signalling molecules as well as induction of expression of c-FOS and NGFI-A. Morphologically, BDNF appears to promote cell survival and modest neurite outgrowth. A similar effect of BDNF was found in the neuroblastoma cells obtained from cases 9 and 15 in our present study. BDNF (NT-4)/trk-B signalling may act in neuroblastomas to promote survival in an autocrine or paracrine manner *in vivo*. The higher levels of expression of *trk*-B in advanced stage neuroblastomas with *MYCN* amplification compared with favourable tumours may contribute to their more aggressive properties. The enhancement of invasion and metastasis by BDNF of some neuroblastoma cell lines may further support this hypothesis [14].

It was surprising that the primary culture cells with *MYCN* amplification from case 8 responded to both NGF and NT-3 by undergoing terminal differentiation and the patient is still alive without disease 31 months later. In addition, transfection of *trk*-A into a neuroblastoma cell line is reported to undergo differentiation in response to NGF [15]. These data

suggest that very high levels of *trk*-A expression may overcome the malignant phenotype of neuroblastomas associated with *MYC*N amplification and corresponding overexpression.

Thus, the primary culture of neuroblastomas to test the responsiveness to neurotrophins may be useful for predicting the prognosis of individual patients.

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