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Short Communication

Immunohistochemical Localization of Trk Receptor Protein in Pediatric Small Round Blue Cell Tumors

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Expression of Trk protein has been documented by Northern analysis in neuroblastomas with good prognosis. To localize the expression of this protein at the cellular level within individual tumors, we adapted a recently characterized pan-Trk antibody for use in formalin fixed, paraffin-embedded tissue. We have examined a group of small round blue cell tumors occurring in children, including both high and low stage neuroblastomas, to assess the presence or absence of Trk expression and its cellular localization. Positive staining for Trk protein was observed in four of four low stage (good prognosis) neuroblastomas, five of five primitive neuroectodermal tumors/Ewing's sarcoma, five of five rhabdomyosarcomas, and no lymphomas. Within the neuroblastomas, expression of Trk protein was most striking in ganglion cells, in which positive cytoplasmic staining was demonstrated regardless of tumor stage. The latter observation may lend further insight into the pathobiology of this malignant childhood tumor. (Am J Pathol 1993, 143:1560–1567)

The family of neuronal growth factors or neurotrophins (NT), including nerve growth factor (NGF), brain-derived neurotrophin (BDNF), and the NT-3,4/5 all play a pivotal role in the differentiation and development of the peripheral and central nervous system.[1–3] The prototypical neurotrophin, NGF, is critically involved in the survival and subsequent differentiation of sympathetic[4] and neural crest-derived sensory neurons.[5] To initiate the differentiation program in neuronal cell types, NGF presumably interacts with two distinctive cell surface receptors, the $p75^{NGFR}$, a member of the TNF/FAS CD40/CD30 family, and the Trk receptor tyrosine kinases.[6–8] Although the function of the p75 neurotrophin receptor ($p75^{NGFR}$) remains unknown, numerous lines of experimental evidence indicate that the $p140^{Trk}$ NGF receptor mediates signal transduction and subsequent cellular responsiveness in the nervous system.[9–12] Two closely related Trk genes, Trk B and Trk C, are expressed in multiple cell types in the central and peripheral nervous system and convey responsiveness to BDNF, NT-4, and NT-3, respectively.[13,14]

The role of NGF in differentiation of sympathetic and neural crest-derived neurons has been correlated with the recent identification of trk mRNA in neuroblastomas (NB), a primitive malignant tumor of neural crest origin occurring primarily in young children. It has been recently demonstrated that stage 1 and 2 NBs had high levels of Trk mRNA, whereas stage 4 had undetectable levels.[15,16]

Because these results were derived from Northern analysis of total mRNA from tumor samples, the cel-

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lular localization of the Trk protein could not be determined. To document expression of the protein within individual cells, we used a recently characterized antibody[17] that recognizes the cytoplasmic portion of human and rat Trk protein (identifying Trk A, B, and C) and using immunohistochemical techniques examined a group of NB and other small round blue cell (SRBC) tumors occurring in the pediatric age group.

Materials and Methods

Cases of NB diagnosed at the Children's Hospital of Boston between 1980 and 1992 were identified. From these, four stage I/II, four stage III, four stage IV, and four treated tumors (stages 3 and 4) were examined. Selection of stage III and IV cases was based on the presence of corresponding cytogenetic data documenting the presence or absence of double minutes. Five rhabdomyosarcomas (two alveolar and three embryonal histology) along with five primitive neuroectodermal tumors/Ewing's sarcomas (PNET/ES) and five lymphomas (three lymphoblastic and two Burkitts) were also identified.

Additional data obtained on the cases of NB included patient age, tumor location, stage, and karyotypic presence of double minutes. Tumor stage was determined by Evan's classification system.[18]

The polyclonal Trk antibody was generated against a fusion protein containing the cytoplasmic domain of human $p140^{Trk}$.[17] However, immunoprecipitation and Western blot analyses on the antibody show that it specifically recognized not only Trk A but Trk B and Trk C in 3T3 fibroblasts transfected with the Trk B or Trk C cDNAs. The methods for the immunoprecipitation and Western blot analyses are in Horvath et al.[17] 3T3 fibroblasts transfected with Trk A cDNA and known to overexpress the protein were used to optimize immunohistochemical staining and served as a positive methodologic control. A mouse melanoma cell line, A875, expressing only the $p75^{NGFR}$ and not the Trk receptor was maintained and used as a negative control.[12] 3T3 Trk A fibroblasts and A875 melanoma cells were processed along with tissue sections for immunohistochemistry. Cells were grown to 60% confluency on glass tissue culture slides, rinsed briefly with phosphate-buffered saline, fixed in 10% buffered formalin for 15 minutes, rerinsed with phosphate-buffered saline, and air-dried before staining.

After review of the H&E stained slides on the study group of cases, a representative block was selected for analysis and 4-μ thick sections were cut. All tissues selected for evaluation were fixed in 10% buffered formalin from 1 to 4 hours and paraffin embedded. After xylene deparaffinization and alcohol rehydration, standard immunohistochemistry was conducted using an avidin-biotin based alkaline phosphatase detection kit (Biogenex Labs., San Ramon, CA). The pan-Trk antibody was titrated for use with paraffin sections, and an optimal dilution of 1:500 was identified. Sections were incubated with the primary antibody for 1 to 2 hours at room temperature. A 10-minute pretreatment with 5% goat serum of some sections was required to reduce background. Normal serum, preimmune serum, and additional related antibodies (ie, anti-p75) were used in place of the primary antibody in the negative and positive controls, respectively.

The proportion of positive ganglion cells/neuroblasts in each of the antibody-stained tissue sections was estimated microscopically by counting the percentage of positively stained cells in ten 40× fields and was categorized according to the following: – (negative), 1+ (1 to 10%), 2+ (11 to 50%), 3+ (51 to 75%), and 4+ (>75%).

Frozen tissue from one of the stage I/II, one of the stage III, and the treated stage 4 NB (identified in Figure 4 by an asterisk) were analyzed using immunoprecipitation and Western blot analyses with the anti-Trk antisera. In brief, tissues were homogenized (Brinkman) for 30 seconds in RIPA buffer containing 1 U/ml aprotinin and leupeptin and 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. After detergent lysis and centrifugation, detergent lysates containing 3 mg of protein were immunoprecipitated using the anti-Trk antisera × 18 hours at 4 C, and complexes were concentrated using protein A sepharose. Immune complexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted on nitrocellulose, and probed with the anti-Trk antisera using ^{125}I protein A for detection of immunoreactive proteins. The exposure time for the autoradiographs was 24 hours. Similarly prepared lysates (300 μg/lane), without the immunoprecipitation step, were examined by Western blot analysis using the anti-Trk antisera.

Results

Characterization of the Pan-Trk Antibody

To evaluate the presence of Trk protein by immunohistochemical means an antibody was generated that recognizes the cytoplasmic portion of the hu-

man Trk protein. This antibody interacts with the three known Trk receptor proteins, ie, Trk A, B, and C, as demonstrated by the autoradiograph of these Trk receptors after immunoprecipitation with the Pan-Trk antibody (Figure 1). Further characterization of the antibody is demonstrated by Western blot analysis of 3T3 cells expressing either the Trk A receptor or the NGFR (p75). As shown in Figure 2, the pan-Trk antibody recognizes only the Trk A receptor protein and not the NGFR (p75) in transfected 3T3 cells. The doublet of proteins at 130 and 110 kd reflects variable glycosylation of Trk (unpublished data). In contrast, an antibody developed against the NGFR (p75) only recognizes the $p75^{NGFR}$ and not the Trk A receptor protein.

Trk Protein in 3T3 Trk A Cells

Trk A positivity was seen as red, coarsely granular to globular staining within the cytoplasm of 3T3 Trk A cells.

A high level of expression of Trk protein was noted within membranous processes and distributed in the cytoplasm in a perinuclear/Golgi distribution (Figure 3A). Cells treated with the preimmune serum alone exhibited no staining. In addition, there was no staining of A875 melanoma cells (p75 positive, Trk A negative).

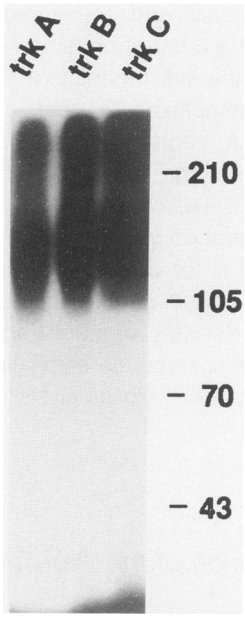

Figure 1. *Autoradiograph of 3T3 Trk A, B, and C cells after affinity cross-linking with ^{125}I NT-3 and immunoprecipitation with the pan-Trk antibody.*

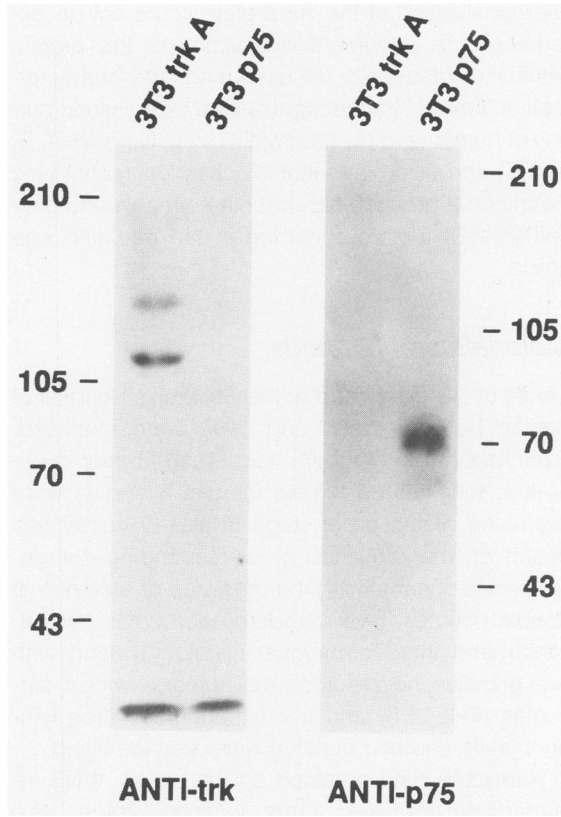

Figure 2. *Specificity of antisera assessed by Western analysis using iodinated protein A.*

Trk Protein in NB Study Group

All stage I/II (4 cases) NBs had moderate to high levels of Trk protein (2+ to 4+) identified by immunohistochemistry. The protein product was localized to the cytoplasm and overlying the nucleus of most neuroblasts. There was focal, weak staining of the accompanying Schwannian stromal components.

The most striking presence of Trk protein was seen within the cytoplasm of mature ganglion cells present in stage III and IV NBs with differentiation (Figure 3B). Again, patchy, weak staining of the stromal (Schwannian) component was observed in most of these tumors. Immature neuroblasts in both stage III and IV NBs were routinely negative. In addition, it was interesting to note that Trk protein was consistently identified within vascular smooth muscle but not in the adjacent nontumorous stromal components (Figure 3B, inset).

The treated tumors that we examined exhibited Trk protein only within ganglion cells. All residual neuroblasts were negative.

Comparison of the Trk protein staining with various parameters including age, site, stage, and the

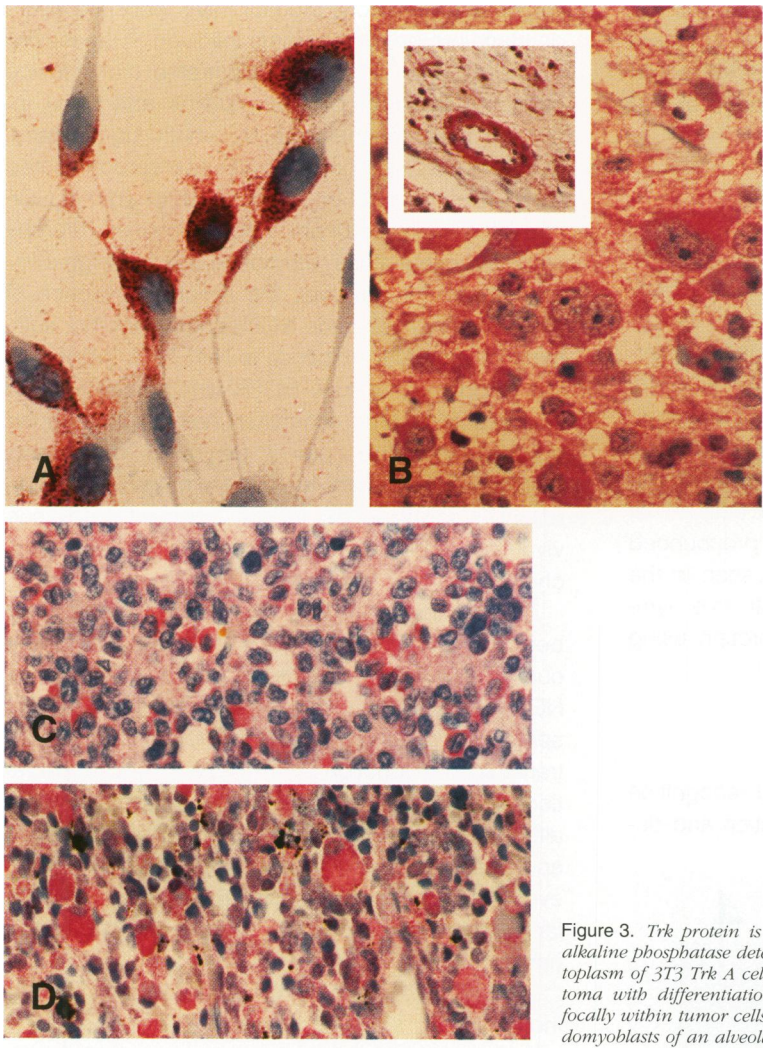

Figure 3. *Trk protein is identified as red deposits using an avidin-biotin/alkaline phosphatase detection system. Trk protein is identified within the cytoplasm of 3T3 Trk A cells* (A)*; within ganglion cells of a stage 3 neuroblastoma with differentiation* (B) *and in vascular smooth muscle* (C, inset)*; focally within tumor cells of a PNET/ES* (C)*; and within differentiating rhabdomyoblasts of an alveolar rhabdomyosarcoma* (D). A, B, C, D = ×400.

presence or absence of double minutes is summarized in Table 1. In summary, all stage I and II NBs examined had high levels of Trk protein and according to karyotypic analysis had no double minutes, whereas all stage III and IV NBs had undetectable levels of Trk protein in cases where double minutes were present. Trk protein was identified only in the higher stage NBs when double minutes were absent. The identification of Trk protein appears to correlate best with double minute status and the presence of differentiation (ie, ganglion cells) rather than age or site. This appears to hold true even with the treated group of NBs. However, as demonstrated in the one example of a treated stage IV NB in which double minutes are present, Trk protein is also identified. Indeed, in this case the protein is found only within ganglion cells.

The immunoprecipitation/Western blot analyses of all three NB (identified in Table 1 by an asterisk)

Table 1. *TRK Protein in Neuroblastomas**

Age	Site	Stage	D_{min}	TRK
Primary tumors				
2 weeks	ABD	I/II	No	3+
5 weeks	ADR	I/II	No	3+
6 weeks	ADR	I/II	No	3+
6 months*	THX	I/II	No	3+
1 year	ABD	III	No	4+
1 year	ABD	III	No	2+
3 years*	ABD	III	Yes	
12 years	ABD	III	No	GC/2+
1 year	ADR	IV	Yes	
2 years	ADR	IV	Yes	
2 years	ADR	IV	Yes	
4 years	THX	IV	No	GC/2+
Treated tumors				
23 months	ADR	IV	Yes	GC/4+
3 years	ADR	III	N/A	GC/3+
6 years	ADR	III	N/A	GC/3+
11 years*	ADR	III	N/A	GC/3+

* NB case study group compared by age, site (THX, thorax; ADR, adrenal; ABD, abdomen), karyotypic presence of double minutes (D MIN); and level of Trk protein (GC staining limited to ganglion cells; N/A, not available). Cases used for concomitant immunoprecipitation and Western blot analysis designated with "*".

revealed a distinct band at 95 kd. The additional higher molecular weight bands illustrated in Figure 4 represent variable glycosylation of the Trk receptor. The 3T3 Trk A cells have a single band at 140 kd.

Trk Protein in Other SRBC Tumors

Trk protein was documented in all five cases of primitive neuroectodermal tumors examined (PNET/ES) (Figure 3C). The protein was localized to the cytoplasm of scattered tumor cells. The adjacent interstitial stromal components were routinely negative, although, as in the NB, staining of vascular smooth muscle was present. The rhabdomyosarcomas were characterized by prominent staining within differentiating myoblasts, including strap cells in all five cases (Figure 3D) and the most pronounced expression of the protein product was seen in the alveolar type rhabdomyosarcoma. All five lymphoma cases were negative for Trk protein using this antibody.

Discussion

Since the identification of the NGF and recognition of its importance in neuronal differentiation and development, numerous studies have focused on understanding the mechanisms involved in its activity. Some of these studies demonstrated that tyrosine phosphorylation of cellular proteins and gene induction were mechanisms by which signal transduction and cellular responsiveness were mediated by NGF.[9–13] In addition, it was established that NGF had biphasic equilibrium binding kinetics, thereby reflecting interactions with both a low and high affinity receptor.[6–8] However, the isolated presence of the low affinity receptor, NGFR (p75), was not sufficient for a cellular response to NGF.[11,19,20] This was not surprising because NGFR (p75) lacks tyrosine kinase activity. More recently, it has become apparent that the Trk receptor is central in the ultimate mediation of NGF activity and promotes such differentiative sequelae as neurite outgrowth, cell survival, neurotransmitter synthesis and regulation, chemotaxis, and cell death.[21–27]

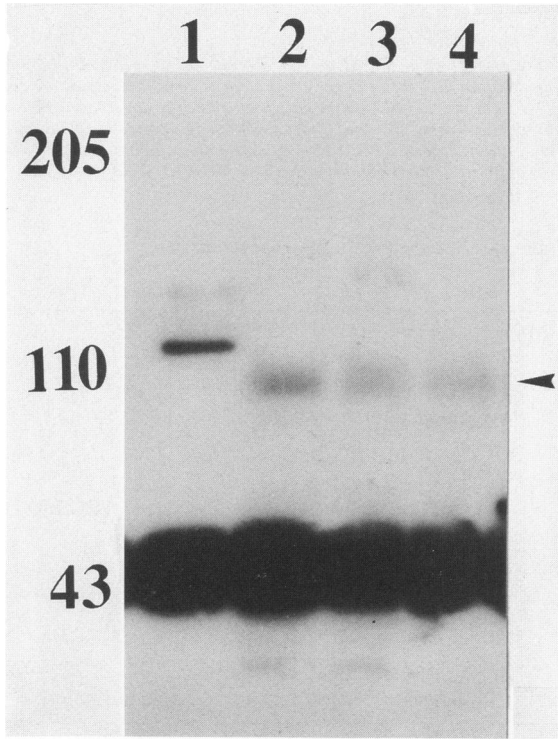

Figure 4. *Autoradiograph of 3T3 Trk A cells, stage I/II, stage III, and treated stage IV NB after immunoprecipitation and probing with the pan-Trk antibody. Lane 1: 3T3 Trk cells; lane 2: stage I/II NB; lane 3: stage III NB; lane 4: stage IV NB (treated).*

The expression of the Trk receptor protein has been documented in both PC-12 cells and in various NB cell lines.[1] In some NB cell lines, addition of NGF variably induces various differentiative responses including neurite outgrowth and neurotransmitter production.[28–30] These *in vitro* observations correlate with the clinical observation that although malignant tumors, some NBs differentiate and may even spontaneously regress.[31,32] However, the mechanisms by which these *in vivo* cellular processes are accomplished are not completely understood.

The expression of the Trk receptor is a potential indication of neurotrophin activity. Nakagawara et al[15,16] recently found that stages I, II, and IVs NBs have increased levels of Trk mRNA. Trk expression also correlated with absence of *N-myc* amplification, although it did not seem to correlate with the degree of differentiation seen histologically. They postulated that increased Trk expression, possibly in conjunction with a functional neurotrophic receptor, somehow enables this subset of tumors (stage I, II, and IV) to spontaneously differentiate or regress. Theoretically, they found that NBs form a spectrum, ranging from the most undifferentiated NBs, which are characterized by high *N-myc* levels, low Trk expression, and little $p75^{NGFR}$ activity to the most differentiated NBs (ie, the ganglioneuroblastomas and ganglioneuromas), which are characterized by uniformly high $p75^{NGFR}$ levels. The $p75^{NGFR}$ expression was thought to be a reflection of the prominent Schwannian component, and Trk expression in this latter group of tumors was variable.

Using a recently characterized antibody and standard immunohistochemical staining proce-

dures, we have been able to localize Trk protein within NB, expanding on these initial observations. We found the highest levels of Trk protein within neuroblasts and ganglion cells of stage I and II NBs and foci of differentiation within single cases of stage III and IV NBs. Interestingly, these latter two cases were also the only two higher stage tumors lacking double minutes (correlating with an absence of *N-myc* amplification). Other than the focal areas of ganglion cell differentiation, stage III and IV NBs were characterized by an absence of staining with the pan-Trk antibody. Findings in the group of chemotherapy treated stage III and IV NBs were similar to the untreated cases in that there was positive cytoplasmic staining of ganglion cells and a lack of staining in primitive neuroblasts.

Our findings in Trk expression and karyotypic absence of double minutes in stages 1 and 2 NB are similar to the elevated Trk mRNA and absence of *N-myc* amplification described by Nakagawara et al.[15,16] The observations support the theory that the presence of an intact signal transduction mechanism, including at least in part high levels of expression of Trk protein, may facilitate an appropriate response to NT and thereby cellular differentiation. Indeed, the most striking expression of Trk protein was localized to the cytoplasm of mature, terminally differentiated ganglion cells. Conversely, most ganglion cells in all tumors express Trk protein. However, these observations do not address the question of whether all NBs must differentiate to ganglion cells before regressing, or whether it is possible that some may simply regress as primitive neuroblasts. Similarly, they do not allow us to comment on the possible role of chemotherapy in the differentiation of NBs. The recent discovery of additional NGF-like NT (ie, BDNF, NT-3,4/5 33–35, and identification of other members of the Trk family, ie, p95, p145 Trk B, and Trk C[13,14,36]) have made it possible to begin to examine neurotrophin signaling in detail and may help to further clarify the role these factors most probably play in the differentiation of NBs.

Results from the immunoprecipitation and Western blot analyses of representative tumor samples demonstrates that the anti-Trk antisera is specific and recognizes Trk receptor protein. We identified a 95-kd truncated form of the Trk receptor protein (in addition to various glycosylated forms) from a stage I and II, III and a treated stage IV NB. This is of interest in that the stage III NB (Table 1 * case) was correspondingly negative by standard immunohistochemistry. Possible explanations for this observation are that: 1) the methodology of immunohistochemistry is not sensitive enough to accurately evaluate all forms (including isoforms) of Trk protein, 2) antigenic sites for Trk are masked during formalin fixation/processing to paraffin, 3) antigenic sites for Trk are exposed during biochemical manipulation, or 4) intratumor variations in Trk expression. Whether the truncated receptor protein has a role in the differentiation and/or behavior of NBs is currently not known; however, its presence further complicates the issues of differentiation and the response to NT.[37–39] Indeed, in recent papers on Trk expression in NB,[15,16] a certain percentage of the stage III and IV NBs examined had moderate to high levels of Trk mRNA expression; however, the form of Trk protein being expressed was not determined.

The PNET/ES are interesting in that slight, focal cytoplasmic expression of Trk protein was identified in scattered cells of all tumors (Figure 3C). Thomson et al[40] reported on a PNET/ES cell line that responded to NGF by using high affinity receptors as a functional signal pathway similar to the NGF receptors on PC12 cells. They hypothesized that not only are these tumors neuroectodermal in origin but that they potentially could respond to NGF at some point during their evolution. Recently, Baker et al[41] described a central nervous system PNET that contained NGF receptor-positive cells. The receptors were functional in that NGF induced the expression of the protooncogene, c-*fos*. These observations and the identification of Trk protein in these tumors correlate with an immunohistological survey of 35 PNETs in which 13 contained NGF receptor-positive cells,[41] suggesting a role for NGF in the therapy of PNET/ES. The description of Trk protein expression in these tumors has not been observed previously. From the information on NGFR studies it seems obvious that Trk protein will play some role in the pathogenesis of PNETs and possibly their differentiation.

There is little information available on the presence of NGF or its associated proteins in lymphomas and rhabdomyosarcomas. Trace amounts of immunoreactive NGF receptors have been demonstrated in a rhabdomyosarcoma cell line[41] and we observed diffuse and extensive Trk expression in all five rhabdomyosarcomas we examined. The degree of expression roughly seemed to correlate with the degree of cellular differentiation (Figure 3D). Whether a NT-activated Trk family member is involved in skeletal muscle differentiation or whether a Trk receptor may use another extracellular polypeptide remains to be determined.

We did not find Trk protein in any of the lymphomas that we examined. This may mean that our

techniques are not sensitive enough to identify the protein and that additional manipulations may be required to expose the epitope for Trk protein in lymphocytes. The evidence for an NGF receptor within the immune system has been alluded to and some researchers have reported that thymocytes contain NGF receptors.[3] However, further study would be required to completely evaluate the signaling mechanism in lymphocytes with respect to NT and Trk protein.

Finally, in the process of these investigations, we noted specific staining of arterial and venous smooth muscle vasculature in all tissues. Recently, Scarisbrick et al[42] identified the expression of NGF, BDNF, and NT-3 mRNAs in the heart and great vessels of the postnatal and adult rat. Further results by Donovan et al (in abstract) have demonstrated by Western blot the presence of Trk protein in smooth muscle from the rat aorta. Further work will be needed to evaluate the function of both Trk and NT in the development of the vascular system and their possible role in the pathogenesis of myogenic tumors.

In conclusion, we have demonstrated the presence of Trk protein in NB (particularly within ganglion cells), PNET/ES, and rhabdomyosarcoma. Although obviously not useful from a diagnostic standpoint, it raises several issues regarding the pathobiology of these tumors. Because the antibody used in this study recognizes Trk A, B, and C, it will be necessary to assess in further studies the specific Trk family member that is involved in the differentiation process of NBs and other pediatric SRBC tumors.

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