Trk Receptor Expression and Inhibition in Neuroblastomas

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Abstract Neuroblastoma, the most common and deadly solid tumor in children, exhibits heterogeneous clinical behavior, from spontaneous regression to relentless progression. Current evidence suggests that the TRK family of neurotrophin receptors plays a critical role in these diverse behaviors. Neuroblastomas expressing TrkA are biologically favorable and prone to spontaneous regression or differentiation, depending on the absence or presence of its ligand (NGF) in the microenvironment. In contrast, TrkB-expressing tumors frequently have MYCN amplification and are very aggressive and often fatal tumors. These tumors also express the TrkB ligand (BDNF), resulting in an autocrine or paracrine survival pathway. Exposure to BDNF promotes survival, drug resistance, and angiogenesis of TrkB-expressing tumors. Here we review the role of Trks in normal development, the different functions of Trk isoforms, and the major Trk signaling pathways. We also review the roles these receptors play in the heterogeneous biological and clinical behavior of neuroblastomas, and the activation of Trk receptors in other cancers. Finally we address the progress that has been made in developing targeted therapy with Trk-selective inhibitors to treat neuroblastomas and other tumors with activated Trk expression.

Background

Neuroblastoma, a tumor of the sympathetic nervous system, is the most common extracranial solid tumor in childhood. Some infants experience regression of their disease without therapy, whereas other patients have maturation of their tumor into benign ganglioneuromas. Unfortunately, the majority of patients have metastatic disease, and many progress relentlessly despite intensive multimodality therapy. Although there have been dramatic improvements in the cure rate for many other pediatric neoplasms, the survival rate for patients with neuroblastoma has lagged behind. However, recent advances in understanding the molecular pathogenesis of neuroblastoma have provided considerable insights into the genetic and biochemical mechanisms underlying these seemingly disparate behaviors (1).

The Trk (NTRK) family of neurotrophin receptors plays a critical role in development and maintenance of the central and peripheral nervous system. This homologous family of tyrosine kinase (TK) receptors consists of TrkA (NTRK1), TrkB (NTRK2), and TrkC (NTRK3). The primary ligands for these receptors are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT3), respectively; neurotrophin-4/5 (NT4) functions through TrkB (2–7). Although TrkC is the primary receptor for NT3, it also binds and activates TrkA and TrkB. Another transmembrane receptor (P75) binds all the neurotrophins with low affinity, and it may contribute to the formation of high-affinity receptors, or otherwise alter the function of Trk receptors (8, 9).

Trk Family Gene Expression in Normal Neuronal Development

Trk expression is crucial for the normal development of the peripheral nervous system. Knockout mice for TrkA, TrkB, or TrkC display unique but overlapping patterns of abnormalities involving the central and peripheral nervous system, but the TrkB knockout is the most severe (10–12). Nevertheless, TrkA expression is most important for the development of normal sympathetic neurons (13, 14). Indeed, NGF is necessary for the survival and differentiation of sympathetic neurons both in vitro and in vivo (15). Sympathetic neurons in newborn animals treated with NGF undergo hypertrophy, whereas neutralizing antibody to NGF results in extensive neuronal cell death. Trk knockouts reveal that TrkB signaling is required in early stages of development of sensory neurons, whereas TrkA expression and NGF dependence occurs later in development (16). For sympathetic neurons, TrkC (±TrkA) is expressed early, TrkB
expression is rarely if ever seen (17, 18), and TrkA expression predominates in later stages. Therefore, the sequential Trk expression TrkC → TrkC + TrkA → TrkA may be important for complete differentiation of normal sympathetic neurons, and the Trk gene(s) expressed reflect the stage of neuronal differentiation of that lineage.

Trk Family Receptors Have Characteristic Features of Transmembrane TK Receptors

TrkA, TrkB, and TrkC have considerable structural similarity (13). Excluding signal sequences, peptide sequence identities between TrkA and TrkB, TrkA and TrkC, and TrkB and TrkC are ~49%, 51%, and 55%, respectively (19). The carboxy region of the extracellular domain contains two immunoglobulin-like domains that confer ligand-specific binding specificity (20, 21), with contributions from leucine-rich motif 3 (22). The peptide sequence in the extracellular domain is 33% conserved between TrkA and TrkB, and the intracellular domain is 67% identical. The TK domain is the most highly conserved among the Trk proteins (71% identity for all three, 88% for TrkA and TrkB), and all three Trk receptors have a 15-amino acid carboxy-terminal tail (19).

The Trk signal transduction system (Fig. 1) involves several pathways characteristic of TK receptors and has been extensively characterized in the rat pheochromocytoma line PC12. NGF binds to TrkA receptors and induces differentiation into sympathetic ganglia. Ligand binding causes receptor homodimerization, which activates the TK and leads to transphosphorylation on at least five tyrosine residues (23, 24). Kinase activity is maximal by 5 to 10 minutes following NGF binding, with subsequent reduction over time due to dephosphorylation or receptor internalization. Autophosphorylation provides docking sites for downstream effectors through SH2 domains. Activation of TrkA induces the phosphorylation and activation of SHC, PI3K, and PLCγ1, which are the primary effectors of Trk activity in NGF-treated PC12 cells (25). Ras/MAPK and AKT are activated downstream of these pathways. Tyrosine phosphorylated SHC associates with the adapter protein GRB2, which in turn binds the SOS-Ras guanine nucleotide exchange factors. SOS enhances the rate of GDP-GTP exchange on Ras, leading to Ras activation. Ras sequentially activates a series of kinases, including RAF1, MEK (MAPK kinase), ERK (MAPK), and RSK. Both MAPK and RSK translocate to the nucleus to participate in the activation of transcription factors that regulate NGF-inducible genes, resulting in survival and neuronal differentiation (Fig. 1).
Other signaling proteins important for normal biological response to ligand binding include SH2B/APS, FRS2 (SNT), and AKT (25). SH2B and FRS2 activation are important for neuronal differentiation, whereas AKT activation is important for survival. SH2B and APS bind Grb2, activate the Ras-MAPK pathway, and can induce differentiation in NGF unresponsive PC12 cells (26). SH2B binding is independent of SHC and PLCγ1 docking, suggesting it may activate a novel signaling pathway (27). Several genes induced by NGF binding to TrkA are known, but many remain to be discovered. However, activation of Trk receptors also may have different consequences, depending on the cellular context. For example, exposure of

Fig. 2. TrkA, TrkB, and TrkC isoform expression and activation. The major isoforms of Trk receptors are the result of alternative splicing. A, activation of TrkA-I (neuronal) or TrkA-II (nonneuronal) isoforms by NGF leads to TrkA activation and signaling, ultimately resulting in survival and neuronal differentiation. In the absence of ligand, alternative pathways are activated leading to apoptosis. The TrkA-III isoform is constitutively active, ligand independent, and promotes survival mainly through the PI3K-AKT pathway. B, there are two full-length TrkB isoforms containing an intact kinase domain, and they are activated in neuroblastomas by endogenous expression of BDNF. There are at least two major subtypes of truncated, kinase-deficient TrkB receptor. One is truncated just beyond the transmembrane domain (TrkB-T1), and the other is slightly longer and includes the SHC binding site (TrkB-SHC). Both contain the ligand binding site, so they may inhibit activation either by competing for ligand binding or by forming functionally inactive heterodimers with full-length TrkB. C, there are two major kinase-active TrkC isoforms that have different C-termini. Signaling from exogenous NT3 promotes survival and differentiation. There are at least four truncated isoforms that lack an intact kinase domain, although the isoform TrkC-T1 is predominant. All are thought to function as decoy receptors by mechanisms similar to the truncated TrkB receptors.
rat PC12 pheochromocytoma cells to NGF causes neuronal differentiation, but NGF exposure of mouse NIH-3T3 fibroblasts transfected with TrkA leads to enhanced cell proliferation (28).

### Isoforms of the Trk Receptors

TrkA, TrkB, and TrkC have distinct isoforms that affect the function of the receptors. The major isoform of TrkA (TrkA-II) expressed in most nonneuronal cells is 796 aa, whereas the neuronal TrkA isoform (TrkA-I) lacks exon 9 (790 aa) (Fig. 2A) (29). Exon 9 encodes 6 aa in the extracellular domain near the transmembrane region in TrkA and does not alter the reading frame. This does not affect NGF binding or receptor activation, but the TrkA-II isoform has enhanced responsiveness to NT3 (30). There is also an early developmental form (TrkA-III) that splices out exons 6, 7, and 9 and is constitutively active independent of ligand (31). TrkB is expressed as both full-length (kinase-intact) and truncated (kinase-deleted) isoforms (Fig. 2B) (19, 32, 33). There are two kinase-intact isoforms that differ by the presence or absence of exon 16, but the reading frame and kinase domain are unaltered. There are at least two kinase-deleted isoforms of TrkB that are truncated just beyond the transmembrane domain and differ by the absence (TrkB-T1) or presence (TrkB-SHC) of the SHC binding site (34). Both may act as dominant-negative inhibitors of full-length TrkB kinase activity, because expression of truncated TrkB receptors inhibits BDNF-induced neurite outgrowth (35). This inhibition may occur by ligand sequestration or by formation of functionally inactive heterodimers. TrkC also has full-length and truncated isoforms, similar to those found for TrkB (Fig. 2C) (36).

### P75 Contributes to Neurotrophin Binding and High-Affinity Receptor Formation

All NGF-related ligands also bind to P75LNTR (P75), which belongs to the tumor necrosis factor receptor superfamily (TNFRSF25). The biological consequences of P75 expression are controversial, but it can mediate apoptosis in developing neurons in the absence of Trk receptors. Structurally, this receptor consists of four cysteine-rich extracellular domains and an intracellular death domain (28, 37). P75 binds NGF and related neurotrophins with low affinity, but its effect on the function of Trk receptors is less clear. Transfection with P75 increases the number of high- and low-affinity NGF binding sites in TrkA-expressing PC12 cells (38). This probably results from a direct interaction with Trk receptors, inducing a conformational change, but P75 may also play a role in ligand presentation (39). P75 can interact directly with TrkA, TrkB, and TrkC in both extracellular and intracellular domains, and this interaction may facilitate functional responses to neurotrophins (9). P75 expression may increase the sensitivity of TrkA to low concentrations of NGF. Furthermore, P75 expression induces apoptosis in the presence of NGF (40, 41), but this apoptotic signaling is inhibited by the presence of TrkA receptors (42). Nevertheless, the effect of P75 on the cellular response to neurotrophins is complex and may depend on the concentration of ligand, the ratio of receptors, the cell type in which it is expressed, and its stage of differentiation (28, 37, 39).

### Clinical-Translational Advances

*Trk family expression and function in neuroblastomas.* TrkA is expressed at high levels in biologically favorable neuroblastomas. We first showed that high TrkA expression in primary neuroblastomas was associated with favorable clinical features and inversely associated with MYCN amplification (43). We (43, 44) and others (45, 46) also showed that TrkA expression is strongly predictive of a favorable outcome. Furthermore, most neuroblastomas express very low or undetectable levels of NGF. Low-stage neuroblastomas usually express high TrkA and respond to NGF by enhanced survival and terminal differentiation (44, 47). In contrast, cells grown in identical media without NGF undergo apoptosis within 7 days. This result mimics the behavior of normal sympathetic neurons placed in culture with or without NGF. TrkA expression is low or absent in most advanced stage tumors, and they do not undergo terminal differentiation in response to NGF, which suggests the NGF/TrkA pathway is responsible for differentiation and regression of favorable neuroblastomas. Human TrkA maps to 1q21, but no mutations or activating rearrangements have been identified to date (48).

TrkB and its ligand BDNF are highly expressed in biologically unfavorable neuroblastomas. Full-length TrkB and BDNF are expressed in more aggressive neuroblastomas and highly correlated with MYCN amplification (P < 0.001) (49), whereas truncated TrkB (lacking a TK domain) is expressed in more differentiated tumors, such as ganglioneuroblastoma. Addition of BDNF to the TrkB-expressing neuroblastoma cell line SMS-KCN caused enhanced cell survival in serum-free media (49). In addition, we have shown that TrkB expression in neuroblastomas is associated with drug resistance and expression of angiogenic factors (50, 51). Others found similar results in SH-SYSY cells that were induced to express TrkB by retinoic acid treatment (52). Thus, the expression of both BDNF and full-length TrkB may represent an autocrine or paracrine survival pathway that is important for the aggressive behavior of some neuroblastomas (52, 53). We cloned human TrkB and mapped it to 9q22 (19), but we have not found mutations or activating rearrangements to date in neuroblastomas.

TrkC is also expressed predominantly in biologically favorable neuroblastomas. We showed that full-length TrkC is expressed in about 25% of primary neuroblastomas, and these tumors essentially represent a subset of the TrkA-expressing tumors (54). These tumors are also associated with younger age and lower stage, and they lack MYCN amplification (54, 55). None of the neuroblastomas tested expressed appreciable levels of NT3 mRNA, so an autocrine or paracrine pathway of activation is unlikely. Human TrkC maps to 15q25, and no genomic rearrangements involving TrkC have been reported.

*Trk family expression and activation in other cancers.* A variety of other cancers have rearrangements or aberrant expression of Trk genes. Trk family gene rearrangements have been identified in papillary thyroid carcinomas, secretory breast cancers, pediatric sarcomas, and leukemias. For example, the TrkA gene is frequently activated in papillary thyroid carcinomas by forming a chimeric receptor gene with several partner genes. Trk receptor activation may result from either intrachromosomal or interchromosomal rearrangement,
juxtaposing the TrkA TK domain to sequences from different genes such as TPM3, TPR, TFG, tropomyosin, and others (56, 57). Similarly, congenital (infantile) fibrosarcoma and mesoblastic nephroma are characterized by t(12;15)(p13; q25) translocation, which results in a TrkC-ETV6 chimeric gene and protein (58, 59). A similar TrkC-ETV6 translocation is also found in secretory breast cancers (60) and some cases of acute myelogenous leukemia (61, 62). Thus, translocations or rearrangements of Trk family genes result in several different chimeric genes and proteins that have constitutive activation of the kinase, and this in turn contributes to oncogenic transformation in a variety of cancer types.

Aberrant expression of unarranged Trk genes may also play a role in the pathogenesis of many other tumor types in addition to neuroblastoma. For example, TrkB is overexpressed in unfavorable Wilms tumors, similar to neuroblastomas (63), and TrkC is overexpressed in favorable medulloblastomas in a manner very analogous to TrkA expression in neuroblastomas (64). Trk genes are also aberrantly expressed in medullary thyroid carcinomas (65), prostate cancers (66, 67), breast cancers (68, 69), and others. Thus, rearrangement or aberrant expression of Trk genes clearly plays an important role in a variety of cancers, so a better understanding of the expression and function of Trk genes and proteins may facilitate targeting these receptors with therapeutic agents.

Trk inhibition in the treatment of neuroblastomas and other Trk-expressing tumors. Given the apparent roles of Trk genes in the biological and clinical behavior of neuroblastomas, inhibiting Trk receptors may be an important adjunct to therapy. Indeed, drugs that target Trk receptors should be more specific and less toxic than conventional chemotherapy. Lestaurtinib (CEP-701) is a Trk-selective TK inhibitor at nanomolar concentrations that blocks Trk activation by ligand. We have shown that precursor compounds to Lestaurtinib have efficacy in treating neuroblastomas in preclinical xenograft models (70, 71). Furthermore, Lestaurtinib is currently in a Phase I clinical trial in neuroblastoma patients. Based on our preclinical studies, it is likely that Trk inhibitors will be most effective in combination with conventional agents in the treatment of high-risk neuroblastomas. Presumably this agent would block an important survival pathway, rendering tumor cells more susceptible to cytotoxic drugs, as suggested by our preclinical studies (51). Trk inhibitors may also be useful, alone or in combination, in the treatment of more favorable tumors by activating pathways of developmentally programmed cell death. Thus, based on apparent efficacy and limited toxicity, Trk-targeted therapy will likely become an important adjunct to neuroblastoma therapy.

Trk-targeted kinase inhibition may also be useful in the treatment of other types of cancer that overexpress Trk receptors, such as prostate, pancreatic, and breast cancer. Lestaurtinib (CEP-701) and the prodrug CEP-2563 have been used in clinical trials for adults with solid tumors (72–74). Interestingly, Lestaurtinib can also inhibit the FLT kinase at similar concentrations, and FLT kinase is activated by mutation of internal tandem duplication in some acute leukemias in children and adults. Thus, Trk-targeted inhibitors may be important for treating a number of pediatric and adult cancers, but we will have to remain cognizant of off-target effects.

Conclusions

It is clear that TrkA and TrkB play important roles in the biological and clinical behavior of neuroblastomas. Absent expression or abnormal function of Trks may render cells unable to undergo differentiation in response to ligand in their microenvironment, so they would continue to grow when they should differentiate, and survive when they should die. The delayed activation of these normal developmental pathways could explain the spontaneous regression of neuroblastoma seen in infants, or the spontaneous maturation to benign ganglioneuromas seen in older patients. Conversely, the coexpression of TrkB and its ligand BDNF in unfavorable neuroblastomas represents a potent survival pathway that promotes growth, angiogenesis, and resistance to therapy. The expression of TrkB in developing sympathetic neurons is rare, so TrkB expression in neuroblastomas seems to be aberrant or inappropriate for this cell type. In any case, there is great potential for the use of Trk inhibitors in the treatment of neuroblastomas, as well as other malignancies with activated or overexpressed Trk genes. Not only is there preclinical and clinical evidence of efficacy, but there has been very little toxicity, suggesting a favorable therapeutic index.

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