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Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression

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ABSTRACT **Although initiating mutations in the ret protooncogene have been found in familial and sporadic medullary thyroid carcinoma (MTC), the molecular events underlying subsequent tumor progression stages are unknown. We now report that changes in trk family neurotrophin receptor expression appear to be involved in both preneoplastic thyroid C cell hyperplasia and later tumor progression. Only a subset of normal C cells expresses trk family receptors, but, in C cell hyperplasia, the affected cells consistently express trkB, with variable expression of trkA and trkC. In later stages of gross MTC tumors, trkB expression was substantially reduced, while trkC expression was increased and often intense. In a cell culture model of MTC, exogenous trkB expression resulted in severely impaired tumorigenicity and was associated with 11-fold lower levels of the angiogenesis factor vascular endothelial growth factor. These results suggest that trk family receptor genes participate in MTC development and progression, and, in particular, that trkB may limit MTC tumor growth by inhibition of angiogenesis.**

The trk family of neurotrophin receptors, trkA, trkB, and trkC, and their neurotrophin ligands, promote the survival, growth, and differentiation of central nervous system neurons and other neural crest-derived cells (1). In cell culture, expression and stimulation of the trk family receptors can result in cell proliferation or differentiation, depending on the cell type. Expression of specific trk family members plays an important role in several human cancers. For two types of cancer, trk family expression is correlated with disease progression. In neuroblastoma, expression of trkA (2, 3) or trkC (4) correlates with good prognosis, and expression of trkB (5) correlates with poor prognosis. Several studies using neuroblastoma cell lines have suggested that trkA expression and activation can result in cell differentiation (6–8), while trkB activation can result in growth stimulation and increased invasion (5, 9). Similarly, in medulloblastoma, trkC expression has been found to correlate with good prognosis (10), and expression of trkC in medulloblastoma cell lines resulted in alterations in morphology consistent with cell differentiation (11).

We have now examined the patterns and biology of trk family receptor expression in medullary thyroid carcinoma (MTC), a cancer that arises from the thyroid C cell. MTC can occur as a sporadic disease or as part of the autosomal dominant multiple endocrine neoplasia type 2 (MEN 2) syndromes (12). There are three related MEN 2 syndromes. In MEN 2A, patients develop MTC, pheochromocytoma, and parathyroid hyperplasia. In MEN 2B, patients develop MTC, pheochromocytoma, and mucosal neuromas. In familial medullary thyroid carcinoma, only MTC occurs. Each of these syndromes results from an inherited activating mutation in the ret tyrosine kinase gene. Like almost all cancers, MTC is a multistage disease. Thus, in the MEN 2 syndromes, the inherited ret mutation predisposes the individual to an initial general hyperplasia of the thyroid C cells; progression to this C cell hyperplasia stage may require genetic lesions in addition to the ret mutation. Subsequently, one or more independent clonal tumors arise from these hyperplastic cells (13, 14), suggesting that additional changes underlie the progression from C cell hyperplasia to MTC tumor formation. Further progression steps in MTC, seen in only a subset of patients, can lead to a more aggressive phenotype in this usually indolent cancer (15). These progression steps probably reflect additional genetic or epigenetic changes that are accompanied by loss of differentiation of the neoplastic C cells. We now show that the patterns of expression of the trk family of neurotrophin receptors change during MTC progression and may play a critical role both in maintenance of the normal C cell phenotype and in driving key stages of progression of MTC.

MATERIALS AND METHODS

DNA Constructs. Expression constructs were made by cloning the coding regions of trkA [from pDM69 (16), a gift of Mariano Barbacid], trkB [from pSLX-trkB (17), a gift of Tony Hunter], and trkC [from pBS-trkC (18)] into the pLNCX retroviral vector (19).

Primary Tissues and Cell Culture. Medullary thyroid carcinomas from 25 patients diagnosed between 1975 and 1993 were obtained from the Johns Hopkins Hospital and Hopkins Bayview Medical Center pathology files. Seven samples of C cell hyperplasia associated with hereditary MTC syndromes and one of reactive C cell hyperplasia associated with papillary thyroid carcinoma were included. Control thyroid tissues ($n = 10$) were obtained from histologically normal areas of thyroid glands that had been surgically removed for nodular hyperplasia or follicular adenomas and from normal autopsy specimens. The tissues were fixed routinely in neutral-buffered 10% formalin and were paraffin embedded.

The TT cell line of human MTC (20) was cultured in RPMI-1640 with L-glutamine containing 16% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were obtained from Regeneron Pharmaceuticals (Tarrytown, NY), Promega, or Sigma. All three neurotrophins were used at a concentration of 50 ng/ml of culture media. For all growth curves, the cells were plated and allowed to reattach to the plate for 2 days. On day 0, cells were either counted or detected by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay according to manufacturer's instructions (Sigma) and then either media containing either the

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Abbreviations: MTC, medullary thyroid carcinoma; MEN, multiple endocrine neoplasia; VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3.

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appropriate neurotrophin or an equal amount of sterile 1× Dulbecco's–PBS without calcium was added to the remaining plated cells. Cells were counted as indicated on the growth curves and the medium was changed every 4 days for a total of 12 days.

Retroviral constructs were packaged according to a published protocol (19). The Psi-2 ecotropic packaging line (19), which was grown in DMEM with 10% fetal bovine serum, was transfected with pLNCX, pLNC-trkA, pLNC-trkB, or pLNC-trkC by calcium phosphate precipitation of the DNA. Supernatant from the transfected Psi-2 cells was used to infect the amphotropic PA317 packaging line, which was also grown in DMEM with 10% fetal bovine serum. The PA317 cells were selected in 0.5 mg/ml G418 and individual clones were obtained and assessed for viral titer and the correct size viral RNA transcripts. Supernatants were used to infect TT cells. Two days after infection, the TT cells were split and selection in 0.5 mg/ml G418 was begun. Both pooled populations of transduced cells as well as individual colonies were obtained as described in *Results*. After selection, cells were maintained in media containing 0.25 mg/ml G418.

Antibodies. Rabbit polyclonal antibodies against human calcitonin were obtained from Signet Laboratories (Dedham, MA). Affinity-purified polyclonal rabbit antibodies against peptide sequences in the carboxyl-terminal regions of trkA, trkB, and trkC were obtained from Santa Cruz Biotechnology. We verified the specificity at the level of immunostaining by performing blocking studies with the immunizing peptides as detailed below. The antiphosphotyrosine monoclonal antibody 4G10 was used according to manufacturer's instruction (Upstate Biotechnology, Lake Placid, NY).

Immunohistochemical Staining. Tissue sections (5 μm) were deparaffinized, incubated with 0.6% hydrogen peroxide in methanol, then permeabilized by incubating in 0.1% saponin (Sigma) for 30 min at room temperature. The sections were then incubated in a blocking solution containing 5% goat serum and 1% BSA TBS (10 mM Tris, pH 7.4, 0.85% NaCl) for 1 hr at room temperature. The sections were then incubated with primary antibodies diluted in TBS with 1% BSA overnight at 4°C. Final antibody concentrations were as follows: trkA, 1 μg/ml; trkB and trkC, 2 μg/ml; and calcitonin, 1:1 dilution of manufacturer's prediluted antiserum. Primary antibody binding was localized by using an avidin—biotin–peroxidase kit (Vector Laboratories) according to the manufacturer's instructions. The sections were then incubated in 0.05% 3,3′-diaminobenzidine (Sigma) in 0.1% hydrogen peroxide to produce a brown reaction product. Sections were counterstained with Mayer's hematoxylin.

Controls for specificity included blocking the primary antibody with either the immunizing peptide or an unrelated peptide or by using 5 μg/ml nonimmune rabbit IgG (Sigma) in place of primary antibody. For peptide blocking, diluted antibody was incubated for 2 hr at room temperature with 10 μg/ml of the appropriate peptide before being applied to the tissue section.

Intensity of staining of each trk receptor in C cells and MTC was graded as strong, moderate, weak, or absent independently by two pathologists (B.K.M. and P.R.M.); these independent assessments did not differ by more than one grading level. Statistical significance of the differences of staining by the same antibody between normal thyroid, C cell hyperplasias, and medullary thyroid tumors was calculated by using a Z value for the difference between proportions (21).

Immunoprecipitation and Western Blot Analysis. Cells were grown to near confluence in a T75 flask. The cells were then incubated in RPMI-1640 with 0.5% fetal bovine serum for 48 hr. The cells were then washed two times with 1× PBS and incubated in RPMI-1640 without serum for 1–2 hr. The serum-free media were then removed and replaced with serum-free media containing either 50 ng/ml of the appropriate neurotrophin or an equal volume of 1× PBS. The cells were incubated at 37°C for 10 min, washed twice in cold 1× TBS, then harvested into lysis buffer (0.5 ml of 1× TBS with 1% Nonidet P-40, aprotinin, PMSF, sodium orthovanadate, pepstatin, and leupeptin), scraped off of the flask, and centrifuged for 10 min in a microcentrifuge. For immunoprecipitations, these supernatants were incubated with 5 μl of anti-pan trk antibody [Trk (C-14) antibody, Santa Cruz Biotechnology] for 2 hr at 4°C, and precipitated with protein A-agarose (Boehringer Mannheim). The samples were separated by SDS/PAGE, electroblotted onto nitrocellulose, and immunostained with either the anti-pan trk antibody or anti-phosphotyrosine antibody (Upstate Biotechnology). The bound primary antibody was detected with appropriate enzyme-conjugated secondary antibody and the complex was detected by using the enhanced chemiluminescence detection system (Amersham) per manufacturer's instructions.

Soft-Agarose Cloning Assay. A bottom layer of 0.8% SeaPlaque agarose (FMC) in TT media was plated in 35-mm gridded cell culture dishes and allowed to harden. TT cells (10^4 per dish) were resuspended as single cells in either control media or neurotrophin-containing media with 0.4% SeaPlaque agarose and plated over the bottom layer. The dishes were allowed to incubate for a few hours, then TT media was layered on the top to prevent drying. Colonies were counted after 1 mo of growth.

Tumor Growth in Nude Mice. Female nude mice at 6–8 wk of age were obtained from the National Institutes of Health (Bethesda, MD). The appropriate TT cell lines were resuspended at a density of 5×10^6 cells/0.1 ml in 1× PBS, then injected into one flank of each mouse. The mice were monitored for tumor development, then measures were taken of the smallest and largest diameters of each tumor as the tumor grew.

Vascular Endothelial Growth Factor (VEGF) Measurement. Cells were plated at 1×10^6 cells per well in 24 well plates. Two days later, cell culture medium was removed, cells were washed with PBS, and fresh cell culture medium was added. After 24 hr, aliquots of medium were assayed for VEGF by ELISA (R & D Systems).

RESULTS

Expression of trk Receptors in MTC

Normal Thyroid. To evaluate trk family receptor gene expression in normal C cells, we studied 10 thyroid glands from subjects without MTC, in which C cells could be specifically identified by calcitonin immunoreactivity (Fig. 1*A*). None of the C cells in any of these samples exhibited any distinct immunostaining for trkA or trkC (Fig. 1 *B* and *D*, summarized in Fig. 2). However, in eight of these 10 glands, trkB immunoreactivity was distinct within subsets of normal C cells (Fig. 1*C*, summarized in Fig. 2). The proportion of trkB-positive calcitonin-positive C cells ranged from 11%–28%, with a mean of 17%. These findings indicate that a subset of normal C cells express trkB but lack trkA and trkC expression (Fig. 1).

No distinct trk family receptor staining was observed in any non-C cell areas of the thyroids, although weak staining of follicular epithelium was seen with all three antibodies (Fig. 1). This weak staining was blocked by the specific epitope peptides; however, it was not observed at higher dilutions of the anti-trkB antibody, whereas the strong C cell staining remained.

C Cell Hyperplasia. In C cell hyperplasia, the first stage of MTC development in the MEN 2 syndromes (22), trkB was consistently expressed. In seven C cell hyperplasias from patients at risk for MEN 2A or familial medullary thyroid carcinoma, distinct trkB immunoreactivity was observed in most of the C cells (Figs. 1*G* and 2). The staining intensity was strong in six cases and moderate in one case (Fig. 2). TrkA and trkC immunoreactivities could also be localized to these hyperplastic C cells in most cases, although the staining intensities were more variable than the consistently strong staining observed with the trkB antibody (Figs. 1 *F* and *H* and 2). We also examined one case of reactive C cell hyperplasia adjacent to a papillary thyroid carcinoma. As in the familial C cell hyperplasias, strong trkB immunoreactivity was seen in almost all of the C cells present, with minimal staining

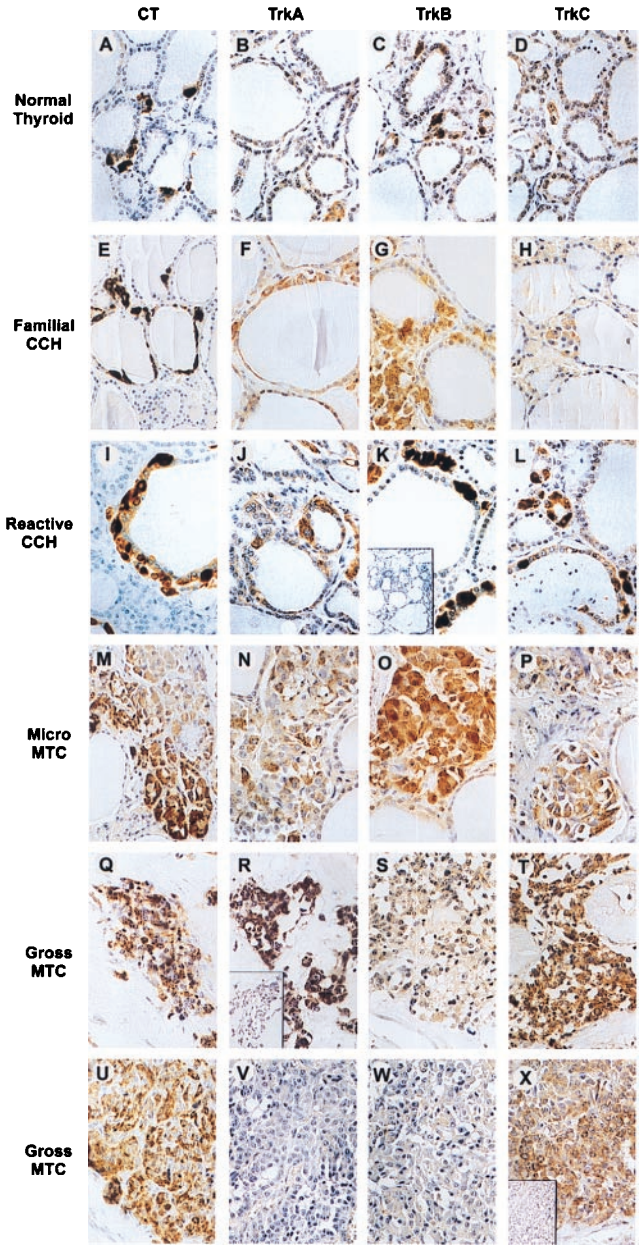

FIG. 1. Immunohistochemical staining of Trk family of receptors in normal thyroid C cell hyperplasia. (*A–D*) A representative normal thyroid with the C cells strongly staining for calcitonin (*A*) and trkB (*C*) with no trkA (*B*) or trkC (*D*) immunoreactivities. (*E–H*) C cell hyperplasia (CCH) from a patient with MEN 2A, stained for calcitonin (*E*), trkA (*F*), trkB (*G*), and trkC (*H*). (*I–L*) A thyroid containing a reactive C cell hyperplasia secondary to papillary thyroid carcinoma. Strong staining of the C cells can be seen with the calcitonin (*I*), trkB (*K*), and trkC (*L*) antibodies and moderate staining with the trkA (*J*) antibody. The inset in *K* is a representative example of the loss of staining observed when the trkB antibody is preincubated with its immunizing peptide. (*M–P*) Microscopic MTC contained within the thyroid of a patient with MEN2A, stained for calcitonin (*M*), trkA (*N*), trkB (*O*), and trkC (*P*). The trkA and trkC antibodies moderately stained both the C cells can be seen with microscopic MTC, while the trkB antibody strongly stained both areas. *Q–T* and *U–X* are two different gross MTC tumors stained for calcitonin (*Q* and *U*), trkA (*R* and *V*), trkB (*S* and *W*), and trkC (*T* and *X*). One tumor was strongly positive for trkA (*R*) and moderately positive for trkB (*S*) while the other exhibited no trkA staining (*V*) and only weak trkB staining (*W*). Both tumors were strongly positive for trkC (*T* and *X*). The insets in *R* and *X* are representative examples of the loss of trkA or trkC staining observed when the respective antibody was preincubated with its appropriate immunizing peptide.

of the surrounding follicular epithelium (Fig. 1*K*). Moderate trkA immunoreactivity was also identified in a smaller proportion of C cells than trkB. Likewise, trkC immunoreactivity was increased. The associated papillary carcinoma was conspicuously negative for all three trk family proteins (data not shown). Thus, progression to hyperplasia may select for a population of C cells, which expresses trkB, or may be accompanied by increased expression of trkB in most C cells.

Medullary Thyroid Carcinoma. During progression from C cell hyperplasia to later stages of MTC, substantial changes were seen in the relative expression of the trk family receptors. We classified MTC cases into stages of microscopic MTC, gross MTC, and aggressive MTC; this latter group was defined by involvement of the tumor in the patient's death. Examples of the trk family immunohistochemistry seen in each of these stages are shown in Fig. 1 *M—X,* and the results are summarized in Fig. 2. Whereas the immunohistochemistry results for microscopic carcinomas were similar to those seen in C cell hyperplasia, the later stages of gross tumors exhibited much less prominent immunohistochemical staining for trkB than was seen in C cell hyperplasia. Thus, in contrast to the consistently strong trkB immunoreactivity observed in C cell hyperplasia, only 1 of 20 gross or aggressive tumors showed strong trkB immunohistochemical staining. This decrease in the proportion of carcinomas staining strongly for trkB vs. the proportion of C cell hyperplasias staining strongly for trkB was statistically significant ($P < 0.000001$).

Second, strong trkC immunostaining, which was never seen in normal C cells, was a characteristic finding in established MTC lesions. Moderate or strong trkC staining was seen in 87% of the MTC tumors (Figs. 1 *T* and *X* and 2). Especially in large tumors, and importantly, those associated with aggressive metastatic disease leading to death of the patients, intense trkC staining could be detected in a large percentage of cells that were negative for trkB (Fig. 1). Similarly, trkA was strongly or moderately expressed in 68% of MTC tumors, although the correlation with aggressive disease was not as prominent as that seen for trkC.

The increase in the proportion of cases with either strong or moderate trkA or trkC staining in tumors, as compared with normal C cells, was statistically significant ($P < 0.0003$) for both trkA and trkC. Thus, at least two changes in trk family expression consistently occur during MTC development and progression. The first of these changes, seen in C cell hyperplasia in the earliest stage of MTC development, involves increased expression of all trk family members. Subsequently, during progression from C cell hyperplasia to palpable tumors, there is a decrease in expression of trkB. These immunohistochemistry results suggest that changing patterns of expression of the trk family members may be important in progression of MTC, and that trkB expression may also be important to the biology of a subset of nonneoplastic C cells.

Effect of trk Family Receptor Expression in Cultured MTC Cells

Expression of the trk Receptors. To further address the biological consequences of trk family receptor expression in MTC, we used an established cell line of human MTC, the TT cell line (20). We first examined these cells for expression of functional trk family receptors and possible response to the trk ligands, by monitoring for autophosphorylation of each receptor in response to each neurotrophin ligand. Cells were treated with NGF, BDNF, or NT-3, immunoprecipitated with an anti-pan trk antibody, and Western blotted for antiphosphotyrosine. On treatment with NT-3, but not NGF or BDNF, TT cells showed a tyrosine phosphorylated protein at about 150–160 kDa, indicating a low but detectable level of functional trkC (Fig. 3). However, treatment of TT cells with NGF, BDNF, or NT-3 had no effect on mitogen-activated protein kinase phosphorylation, cell morphology, growth characteristics, or expression of the calcitonin gene (data not shown).

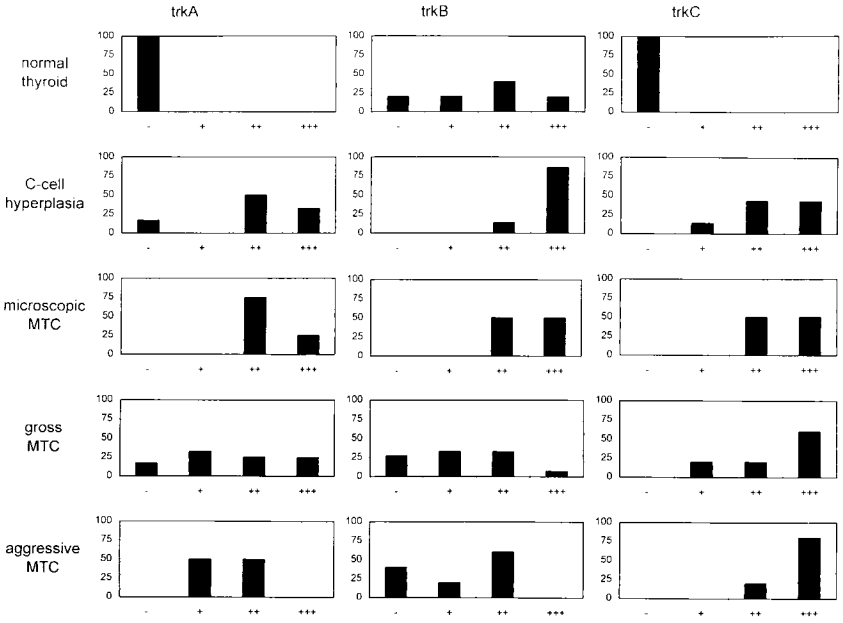

FIG. 2. trk family expression in the progression from normal C cells to MTC. The *y*-axis for each bar graph indicates the percent of samples in each category (normal thyroid, C cell hyperplasia, microscopic MTC, or gross MTC, as designated to the right of each row), which demonstrated strong (+++), moderate (++), weak (+), or absent (−) staining for each trk family member (as designated at the top of each column). Immunohistochemical staining was seen only for trkB in the 10 normal thyroids examined. Immunohistochemical staining for each trk family member was seen in some of the seven samples of C cell hyperplasia associated with MTC, with trkB having moderate or strong staining in all of the samples. All four microscopic MTC tumors stained moderately or strongly for all three trk family members. Only one gross tumor that was not aggressive stained strongly for trkB, and no aggressive tumor stained strongly for trkB or trkA. In contrast, 53% of gross nonaggressive tumors stained strongly for trkC, and 80% of aggressive tumors stained strongly for trkC.

These results suggest that although trkC is expressed in TT cells, the endogenous levels may be too low to affect the cell phenotype.

We next constitutively expressed full-length constructs of either trkA, trkB, or trkC in the TT cell line. After selection of the retrovirally infected cells in G418, pooled populations of cells were studied. The TT-trkA and TT-trkC cell lines expressed the inserted receptor at a high level, and each of these receptors was autophosphorylated even before addition of ligand. A further increase in phosphorylation was seen after addition of the appropriate ligand (Fig. 3). In contrast, we were unable to detect expression of trkB in any pooled populations of infected cells. A few individual clones were obtained, however, which expressed detectable levels of trkB. These TT-trkB clones contained a low but detectable amount of phosphorylated trkB protein before addition of BDNF, and demonstrated a substantial increase in phosphorylated protein after the addition of ligand to the cells (Fig. 3). In parallel, control pLNCX vector-only clones were selected; as expected, these did not exhibit trk family protein phosphorylation on addition of BDNF.

Alterations in Growth of MTC Cells Expressing trk Family Receptors. After demonstrating that each cell line expressed the appropriate functional trk receptor, we examined the growth and differentiation properties of the overexpressing cell lines *in vitro*. The TT-trkA and TT-trkC cell lines exhibited an increased growth rate, as compared with TT–neo control cells, even before ligand addition (Fig. 4). This increased growth apparently reflects the amount of phosphorylated receptor present in the cells before ligand addition. When these same cells were grown in the presence of appropriate ligand, each demonstrated a further increase in growth rate when compared with cells treated with PBS carrier alone. The TT-trkB cells, which did not have as much basal receptor phosphorylation, did not exhibit this increased basal growth rate. However, increased growth was observed when the TT-trkB cells were grown in the presence of the BDNF ligand (Fig. 4).

After ligand addition, the TT-trkB cell lines appeared distinctly different in morphology from the TT-neo, TT-trkA, and TT-trkC cells. This morphologic change was characterized by the cells "rounding up" and becoming less adherent to the cell culture dish (data not shown). Similar rounding of TT cells has been observed during a differentiation response induced by increased signaling via the ras/raf pathway (23, 24). However, other than this morphologic change, we did not find any of the other markers of ras/raf mediated differentiation, such as appearance of neurosecretory granules as visualized by electron microscopy, or

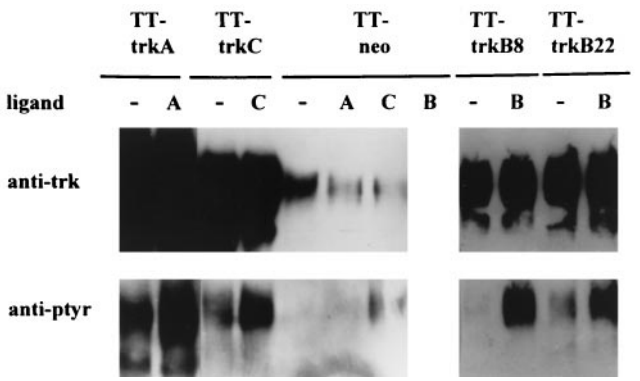

FIG. 3. Immunoprecipitation/Westerns of TT-trk cells. TT cells expressing trkA, trkB, trkC, or an empty vector (neo) were serum starved then treated either with the appropriate ligand (+) or with media containing carrier alone (−). TT-trkA cells were treated with NGF, TT-trkC with NT-3, and TT-trkB with BDNF. The TT-neo cells were treated as indicated. After 10 min of treatment, the cells were harvested and the trk receptor proteins were immunoprecipitated by using a pan-trk antibody. The immunoprecipitated proteins were separated by SDS/PAGE and detected with either the pan-trk antibody or an antiphosphotyrosine antibody. No cell line showed increased tyrosine phosphorylation after incubation with the inappropriate ligand (for example, TT-trkA cells did not show increased phosphorylation after incubation with BDNF) (data not shown).

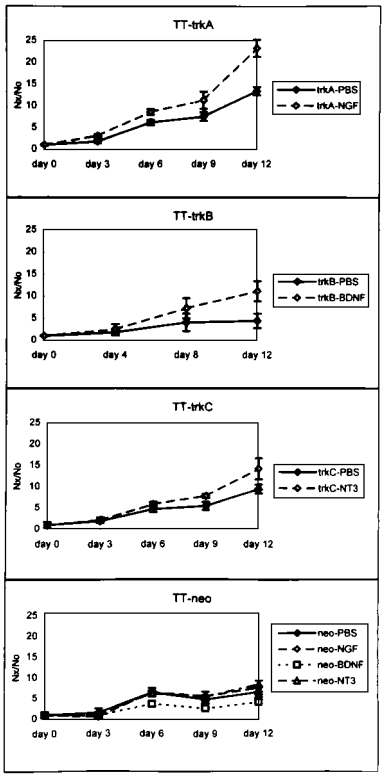

FIG. 4. Growth of the TT cells expressing trk family receptors. Each cell line was grown in media containing the indicated neurotrophin or in media containing an equivalent amount of the neurotrophin solvent, 1× PBS. Each data point represents the number of cells detected at the indicated time point divided by the number of cells detected at day 0 and is an average of several samples tested in at least three independent experiments. The error bars represent plus or minus one standard deviation from the average.

changes in the steady-state level of mRNA from the calcitonin gene and/or in the ratio of calcitonin/CGRP mRNAs, which are produced from the calcitonin/CGRP gene by alternative splicing (data not shown). The TT-trkA and TT-trkC cells also showed no changes in these differentiation features after ligand addition (data not shown).

Alterations in Tumor-Related Properties of the Engineered TT Cell Lines Expressing trk Family Receptors. To further test how the above cellular responses to trk receptor expression might relate to the different patterns of trk expression seen in the natural progression of MTC, we evaluated the tumorigenic capacity of the trk-expressing TT cells in two types of assays. First, the TT-trkA and TT-trkC cell lines demonstrated a 3-fold increase in clonogenicity in soft agar as compared with the TT-neo cell line (Table 1). Second, both the TT-trkA and TT-trkC cells demonstrated increased tumor growth when injected into the flanks of nude mice (Table 1). This increased tumorigenicity was apparent without treatment of the cells with NGF or NT-3, respectively, consistent with the fact that the trkA and trkC receptors are already phosphorylated before ligand addition.

In both of the above assays for tumorigenicity, the TT-trkB cells behaved very differently from the TT-trkA and TT-trkC cells (Table 1). In soft agar, the TT-trkB cells showed no increased cloning efficiency, compared with the TT-neo control cells, with or without ligand addition. Most strikingly, in nude mouse studies, the TT-trkB cells had a markedly diminished incidence of tumor formation. Those few tumors that did grow from TT-trkB cells were barely palpable; they were very flat and adhered tightly to the skin. Moreover, these small TT-trkB tumors had an increased average latency time for appearance of tumor that was 20 days greater than the tumors that grew from TT-neo control cells.

TrkB Expression Suppresses the Secretion of the Angiogenesis Factor, VEGF. Whereas tumor formation and growth was markedly suppressed by expression of trkB in TT cells, growth in culture and soft agar colonization were only slightly affected, suggesting that the most significant role for trkB involves factors necessary only *in vivo*. Furthermore, the TT-trkB tumors that did form were very small and flat, suggesting that these tumors lacked a factor(s) that may allow these tumors to grow beyond a minimum size. It is now well established that tumors must induce neovascularization to grow beyond a minimum size (25). We therefore examined three independent TT-trkB cell clones, two TT-neo clones, and the pooled TT-trkA cells for expression of VEGF, one of the major angiogenesis factors produced by tumor cells (25). By ELISA, TT-trkB cells secrete, on average, 11-fold less VEGF protein than the control TT-neo cells (Table 2). TT-trkA cells secreted about the same level of VEGF as did the TT-neo cells. Together, these data suggest that trkB, via inducing inhibition of angiogenesis, can suppress tumor growth in human MTC.

DISCUSSION

MTC, especially in the hereditary MEN 2 setting, has been shown to be a multistage disease; analysis of the age of onset data for MTC (26, 27) indicates that further genetic or epigenetic lesions must accrue for progression to both the C cell hyperplasia and the carcinoma stages (28). Whereas the hereditary forms of MTC are initiated by a germline mutation in the ret tyrosine kinase gene (29–32), the subsequent genetic lesions underlying MTC development and progression are not known. Our observations, that trkB expression is reduced, and trkC or trkA expression is increased, during MTC tumor progression, suggest that these changes in expression of the neurotrophin receptors may be involved in MTC progression, as they appear to be in neuroblastoma (2–5). One may envision a model in which the increased expression of the trkA or trkC receptor, from the C cell hyperplasia stage and onward, may confer a growth advantage to MTC *in vivo*, as we have shown in MTC cells in culture; this might be similar to the effect of increased erbB-2 expression in breast,

Table 1. Tumorigenicity of MTC cells

	Clonogenicity in soft agar	Mice with tumor/ mice injected	Latency*, days	Average tumor size, mm†
TT-trkA	6.25%	7/9	24.4§	362§
TT-trkB	1.79%	5/39¶	56§	‡
TT-trkC	6.52%	7/9	34.1	548**
TT-neo	2.88%	27/37	36.2	61.2

*Calculated from the time of injection until the appearance of a measurable tumor.
†Calculated volume of the tumor 60 days after injection.
‡Small flat tumors that could not be measured accurately (see text).
§$p < 0.002$ vs. TT-neo.
¶$p < 0.000002$ vs. TT-neo.
**$p = 0.042$ vs. TT-neo.

Table 2. VEGF production by MTC cells

Cell line	VEGF (ng/ml/10^6 cells/24 hr)*
TT-neo	2.85 (1.6, 4.1)
TT-trkB	0.25 (0.07–0.33)
TT-trkA	6.1

*VEGF in tissue culture medium was measured by ELISA.

ovarian, and other cancers (33). By this analogy, specific inhibition of the trkA or trkC receptor may be a candidate for therapeutic intervention in MTC.

In this model, continued expression of the trkB receptor serves to limit tumor growth, and subsequent down-regulation of trkB is required for further tumor progression, as discussed below. Whether these receptors are stimulated by their cognate ligands by endocrine, paracrine, or autocrine means in MTC is uncertain. In the TT cell line and in primary MTC tissue, we can detect mRNA for NGF, BDNF, and NT-3 by reverse transcription—PCR, but not by Northern or Western blotting (data not shown). These results suggest that the levels of the ligands in the tumors may be relatively low; it is unclear whether these low levels of ligand could provide autocrine stimulation.

Our data concerning the dynamics of trkB expression in MTC are particularly intriguing. The finding of expression for this receptor in both a subset of normal C cells and in reactive and neoplastic hyperplastic C cells suggests that trkB is important to the controlled growth of these cells. In this setting, maintaining suppression of VEGF expression would help ensure that dysregulated growth beyond the confines of the location of the normal cells and/or the areas of hyperplasia would not occur. However, once other factors have dictated the conversion of the hyperplastic state toward a malignant state, the suppression of angiogenesis by trkB would not be favorable for tumor growth and the expression of the trkB receptor would be selected against. This would fit well with the low expression levels found in the majority of larger tumors and in the aggressive tumors and with the results we obtained with our trkB-engineered cells in nude mice.

There has been much interest in the mechanisms of regulation of tumor angiogenesis. In numerous tumor types, including MTC, microvessel density correlates with tumor stage and patient prognosis (34). In preclinical models, it has been shown that blocking VEGF or its receptor can block angiogenesis and interfere with tumor growth (25), and several compounds that target this pathway are now in clinical trials. Although we do not yet know the mechanism by which trkB reduces VEGF in MTC cells, there is precedent for decreased VEGF expression in response to neurotrophin receptor signaling. In PC12 pheochromocytoma cells, activation of the trkA receptor by NGF results in decreased VEGF expression (35). Together, these findings suggest that signaling pathways from trkB in MTC cells may provide important leads for the control of tumor angiogenesis.

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