

The Neurotrophin-Trk Receptor Axes Are Critical for the Growth and Progression of Human Prostatic Carcinoma and Pancreatic Ductal Adenocarcinoma Xenografts in Nude Mice

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ABSTRACT

Purpose: Aberrant expression of trk receptor kinases and enhanced expression of various neurotrophins (NTs) have been implicated in the development and progression of human prostatic carcinoma and pancreatic ductal adenocarcinoma. We examined the antitumor efficacy of administration of NT neutralizing antibodies on the growth of established human prostatic carcinoma and pancreatic ductal adenocarcinoma xenografts in nude mice.

Experimental Design: In initial studies, tumor-bearing nude mice were treated with a mixture of NT antibodies [100 µg each of anti-nerve growth factor (NGF), anti-brain-derived neurotrophic factor, anti-NT-3, and anti-NT-4/5] or normal rabbit IgG (400 µg) intratumorally and peritumorally three times/week over a 15-day dosing period. In subsequent studies, tumor-bearing nude mice were treated with individual NT antibodies (100 µg), affinity-purified anti-NGF (0.1, 1.0, or 10.0 µg), or normal rabbit IgG (100 µg) using the same dosing schedule.

Results: Treatment with the antibody mixture inhibited significantly the growth of TSU-Pr1 and AsPC-1 xenografts as compared with IgG-treated controls (maximal inhibition of 53 and 53%, respectively), whereas this treatment caused significant regression in PC-3 xenografts. Treatment of TSU-Pr1 xenografts with either anti-NGF or anti-NT-3 resulted in maximal tumor growth inhibition of 67 and 64%, respectively, whereas anti-brain-derived neurotrophic factor and anti-NT-4/5 did not inhibit tumor growth in this tumor model. Administration of various concentrations (0.1, 1.0, or 10.0 µg) of affinity-purified anti-NGF resulted in maximal TSU-Pr1 tumor growth inhibition of 49, 62, and 66%, respectively.

Conclusions: These data add further support for the therapeutic potential of disrupting trk-signaling events in

select types of nonneuronal human cancers, specifically prostatic and pancreatic carcinomas.

INTRODUCTION

The NT² family of growth factors, NGF, BDNF, NT-3, NT-4/5, and their cognate receptors (trks A, B, C and the low-affinity NGF receptor, p75^{NGFR}) have been implicated in the paracrine growth regulation of a number of neuronal and nonneuronal tumor types. Each NT binds to a specific trk receptor; trkA binds specifically to NGF, trkB binds to both BDNF and NT-4/5, and trkC binds primarily to NT-3. However, NT-3 can bind and activate trkA and trkB as well (1, 2). All NTs bind with various affinities to p75^{NGFR}, a receptor implicated in the regulation of neuronal cell survival, and in the modulation of NT affinity to the various trk receptor subtypes (3, 4). Although NTs aid in the development and maintenance of the adult nervous system, NTs have also been shown to increase tumor invasiveness, enhance clonal growth, and cause changes in cell morphology in a variety of nonneuronal cell types including Wilms' tumor, melanoma, and prostatic, pancreatic, lung, and medullary thyroid carcinoma (5–10). Specifically, human prostatic carcinoma cells have been shown to be chemotactic (10) and invasive (11) in response to NGF *in vitro*. In addition, expression of NGF, BDNF, NT-3, and the trk receptors by immunohistochemical, ELISA, and RT-PCR methods has been demonstrated in human prostatic carcinomas and tumor-derived cell lines (12–18), raising the possibility of a mitogenic role or survival role for NTs in prostatic cancer.

We have demonstrated the expression of the NTs and aberrant overexpression of the trk receptors immunohistochemically and by *in situ* hybridization in human PDAC specimens relative to normal pancreata and in human PDAC-derived cell lines. Furthermore, we demonstrated in a series of Boyden chamber assays that at low nanomolar concentrations of specific NTs (BDNF and NT-3), there was a significant increase in the *in vitro* invasiveness of human PDAC-derived cell lines through growth factor reduced Matrigel (9). In addition, other laboratories have demonstrated NT and trk expression in PDAC-derived cell lines and normal pancreata (19–21) and have shown that NGF expression correlates with perineural invasion and pain associated with PDAC (22). These combined data indicate that NT-trk receptor axes may play a role in the development and progression of both human prostatic and pancreatic adenocarcinoma.

Received 9/6/01; revised 2/18/02; accepted 2/28/02.

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² The abbreviations used are: NT, neurotrophin; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; RT-PCR, reverse transcription-PCR; PDAC, pancreatic ductal adenocarcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

To evaluate the consequences of abrogating NT-trk axes in both prostatic adenocarcinoma and PDAC, we tested the anti-tumor efficacy of the potent trk tyrosine kinase inhibitors, CEP-751 and/or CEP-701, on the development of nine different human and rat models of prostatic carcinoma and in six different human models of PDAC. CEP-751 and CEP-701 were able to exert significant antitumor effects in both human and rat models of prostatic carcinoma independent of growth rate, differentiation state, metastatic ability, or androgen dependence (23, 24). Similarly, administration of CEP-701 inhibited significantly the growth and *in vivo* invasiveness of five of six human PDAC xenografts. In addition, the combination of CEP-701 with gemcitabine resulted in statistically significant potentiation of anti-tumor efficacy relative to CEP-701 and gemcitabine monotherapy in several s.c. PDAC xenograft models (25, 26). Although potent inhibitors of the trk receptor, CEP-701 and CEP-751 also possess inhibitory effects against the vascular endothelial growth factor receptor flk1/KDR/vascular endothelial growth factor-receptor 2, platelet-derived growth factor kinase, and protein kinase C (25). To confirm that the inhibitory effects of CEP-751 and CEP-701 observed in prostatic carcinoma and PDAC xenografts were mediated via NT-trk signaling, we have examined the antitumor efficacy of NT neutralizing antibodies on the growth of prostatic carcinoma and PDAC xenografts in nude mice. Other laboratories have demonstrated significant antitumor efficacy via inhibition of critical growth factor/receptor axes using antibody-mediated approaches. For example, the use of an immunoneutralizing antibody against vascular endothelial growth factor has been reported to prevent primary tumor growth of glioblastoma, rhabdomyosarcoma, and prostate, colon, and gastric carcinomas in nude mice (27–29). In addition, antibodies against the epidermal growth factor receptor have been demonstrated to prevent the formation of A431 human epidermoid tumors in nude mice and cause the complete regression of established A431 xenografts (30). Herceptin (anti-Her-2) administration has demonstrated significant antitumor efficacy preclinically (31) and is currently under clinical evaluation in patients with Her-2-positive breast cancer (32, 33). These combined data support the utility of inhibiting growth factor/receptor axes by the administration of neutralizing antibodies.

Using models shown previously to be growth inhibited by the trk kinase inhibitors CEP-701 and/or CEP-751, in this report we provide further experimental evidence for the involvement of NT-trk axes in the growth of prostatic carcinoma and PDAC xenografts and implicate NGF and NT-3 as specific NTs essential for the growth of TSU-Pr1 prostatic tumors. The data presented add further support for the therapeutic potential of disrupting trk-signaling events in selected types of nonneuronal human cancers.

MATERIALS AND METHODS

Neutralizing Antibodies and Characterization of Activities. The following polyclonal NT antibodies were used: anti-NGF (PeproTech 500-P85), anti-BDNF (PeproTech 500-P84), anti-NT-3 (PeproTech 500-P82), anti-NT-4/5 (PeproTech 500-P83), and affinity-purified anti-NGF (PeproTech 500-P85, affinity purified). The polyclonal antibodies were produced in

rabbits from the corresponding recombinant human NT. The resulting antiserum was purified by ammonium sulfate precipitation, followed by ion exchange chromatography. Normal rabbit IgG (PeproTech 500-P00 for Figs. 1, 3, and 4; R&D AB-105-C for Fig. 5) was administered as a negative control. The antibodies and normal rabbit IgG were resuspended in sterile PBS. We have done extensive testing on the neutralizing capacity of these NT antibodies by demonstrating inhibition of NT-stimulated trk autophosphorylation (data not shown). Using stably transfected NIH3T3-trkA, NIH3T3-trkB, or NIH3T3-trkC cells, we evaluated the effects of NT antibodies (0.001–100 ng/ml in log increments), the minimal concentration of the polyclonal anti-NGF, anti-BDNF, anti-NT-4, or anti-NT-3 that reduced specific NT-stimulated trkA, trkB, or trkC autophosphorylation by >50% was 10, 10, 100, or 100 μ g/ml, respectively. On the basis of densitometric analysis, we have determined that these concentrations of antibody reduced trk autophosphorylation below the minimal detectable level ($\geq 100\%$) in the case of anti-NGF, 84% for anti-BDNF, 65% for anti-NT-4, and 69% for anti-NT-3. Affinity-purified anti-NGF (0.01 μ g/ml) reduced NGF-stimulated trk autophosphorylation 93% relative to control normal rabbit IgG.

Cell Lines. The human prostate carcinoma cell line PC-3, the human prostatic/bladder carcinoma TSU-Pr1, and the human ovarian carcinoma cell line SK-OV-3 were grown in DMEM, RPMI 1640, or McCoy's 5a media, respectively (Cellgro/Mediatech, Washington, DC) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The human pancreatic carcinoma cell lines AsPC-1 and CFPAC were grown in RPMI 1640 or DMEM, respectively (Cellgro/Mediatech) containing 10% fetal bovine serum (Atlanta Biologicals). All cell lines were cultured at 37°C in a humidified incubator with 95% air/5% CO₂ atmosphere.

Animals. Female athymic *nu/nu* mice (8–10 weeks of age; Charles River, Raleigh, NC) were maintained at five/cage in microisolator units. Animals were given a commercial diet and water *ad libitum*, housed at 48% \pm 2% humidity and 22 \pm 2°C, and the light-dark cycle was set at 12-h intervals. Mice were quarantined for at least 1 week before experimental manipulation. Mice weighed between 22 and 25 g on the day of inoculation of tumor cells. All of the animal experiments were performed at Cephalon, Inc., under protocol approved by the Institutional Animal Care and Use Committee.

Tumor Cell Implantation and Antibody Administration. Exponentially growing cells were cultured as described above, harvested, and injected (5×10^6 cells/mouse) 1:1 with Matrigel (Fisher Scientific, Malvern, PA) into the right flank of nude mice. Tumor-bearing animals were randomized according to tumor size into the appropriate number of experimental groups with 8–10 mice/group. All antibodies and control IgG were administered once a day, three times/week, in a total volume of 100 μ l of sterile PBS, with 50 μ l divided among five intratumoral injection sites and 50 μ l divided among five peritumoral injection sites. Antibodies were administered intratumorally and peritumorally to insure local delivery of antibodies close to the tumor site, because antibody stability after systemic administration was not known.

Antibody Mixture Experiments. Nude mice bearing established ASPC-1, CFPAC, SK-OV-3, PC-3, or TSU-Pr1 xe-

nografts received a mixture of NT neutralizing antibodies (400 μg) composed of 100 μg each of anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5. An additional experimental group of nude mice bearing PC3 xenografts received a mixture of NT neutralizing antibodies (100 μg) composed of 25 μg each anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5. Control groups received normal rabbit IgG (400 or 100 μg).

Individual Antibody Experiment. Nude mice bearing established TSU-Pr1 xenografts were randomized into the following experimental groups: (a) normal rabbit IgG (400 μg); (b) a cocktail of NT antibodies (100 μg each anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5); (c) anti-NGF (100 μg) + normal rabbit IgG (300 μg); (d) anti-BDNF (100 μg) + normal rabbit IgG (300 μg); (e) anti-NT-3 (100 μg) + normal rabbit IgG (300 μg); and (f) anti-NT-4/5 (100 μg) + normal rabbit IgG (300 μg). The 300 μg of normal rabbit IgG was added to the individual antibody preparations to keep the total amount of IgG constant.

Affinity-purified Antibody Experiment. Nude mice bearing established TSU-Pr1 xenografts were treated with various concentrations (0.1, 1.0, or 10.0 μg) of affinity-purified anti-NGF or polyclonal anti-NGF (100 μg). The control group was treated with normal rabbit IgG (100 μg).

Tumor Measurements. Tumors were measured using a Vernier caliper every 2–3 days. Tumor volumes were calculated using the following formula: $V(\text{mm}^3) = 0.5236 \times \text{length}(\text{mm}) \times \text{width}(\text{mm}) [\text{length}(\text{mm}) + \text{width}(\text{mm})/2]$. The tumors in each animal were individually normalized to their size at the start of the experiment, and the data were calculated as the change in tumor volume relative to the day 1 volume, using the following formula: relative tumor volume = V_x/V_o , where V_x is the tumor volume at any time point and V_o is the tumor volume at the initiation of dosing (day 1; Refs. 13, 24). For each experimental group, the mean relative tumor volumes and standard errors were calculated (SigmaStat; Jandel Scientific, San Rafael, CA). Statistical analyses were calculated by the Mann-Whitney Rank Sum test (SigmaStat) with $P \leq 0.05$ deemed significant.

RT-PCR Detection of trkA and trkC. Total RNA was isolated from cell lines using the RNAwiz (Ambion). DNA was synthesized from 1–5 μg of RNA using oligo(dT) and a reverse transcriptase kit (Life Technologies, Inc.). The PCR cycle for trkA consisted of 1 min at 95°C, 1 min at 66°C, and 2 min at 72°C. The PCR cycle for GAPDH consisted of 1 min at 95°C, 1 min at 63°C, and 2 min at 72°C. Samples (10 μl) were electrophoresed on a 10% TBE Novex gel (Novex, Inc.) for 1.5 h at 150 V. The PCR mixture included 1.5 μCi of [α - ^{32}P]dCTP (NEN), and the incorporation of label was detected using a PhosphorImager (Molecular Dynamics).

For human trkA, the sense primer (5'-TCCGCCTCCATCATGGCTGCCTT-3') and antisense primer (5'-CCCAAAC-TGTTTCTCCGTCCACA-3') corresponded to nucleotides 1231–1253 and 1426–1449, respectively, of the human trk proto-oncogene insert of Plm6 (13). For human trkC, the sense primer (5'-TTCGCATGAACATCAGTCAGTGTG-3') and antisense primer (5'-CTCACCACACGTGGGGGATAGTAGA-3') corresponded to nucleotides 753–777 and 1057–1881, respectively, of the human trkC cDNA (13). For human GAPDH, the sense primer (5'-ACCACAGTCCATGCCATCAC-3') and antisense primer (5'-TCCACCACCTGTTGCTGTA-3') cor-

responded to nucleotides 586–734 and 998–1037, respectively, of the human GAPDH insert of G3PDH. Oligonucleotides were synthesized by Life Technologies, Inc. (trkA) and Oligo Etc. (GAPDH). All primers used in these experiments are known to cross an intron-exon boundary.

RESULTS

Effects of NT Neutralizing Antibody Mixture on s.c. Prostatic and Pancreatic Carcinoma Xenografts in Nude Mice.

The antitumor efficacy of a mixture of NT neutralizing antibodies [100 μg each anti-NGF, anti-BDNF, anti-NT-3 and anti-NT-4/5 (hereafter referred to as anti-NT antibody mixture)] was determined using two poorly differentiated, androgen-independent, prostatic carcinoma xenografts (TSU-Pr1 and PC-3) and one moderately well-differentiated PDAC xenograft (AsPC-1). Two xenograft models, CFPAC and SK-OV-3, shown previously to be unresponsive to CEP-701 or CEP-751 treatment were used as negative controls (13, 25). The expression of the various NTs and corresponding receptors has been evaluated on the cell lines used in these studies. The PC-3 cell line has been demonstrated to express trkA and trkC but not trkB or the low-affinity NGF receptor p75^{NGFR}. In addition, this cell line expresses both BDNF and NT-3 (11, 13, 15). Similarly, TSU-Pr1 cells express trkA but not p75^{NGFR}; however there are conflicting reports on the expression of trkB and trkC in this cell line (11, 13, 15). TSU-Pr1 cells express NGF, BDNF, and NT-4/5 but not NT-3 (11, 13, 15). Using a pan-trk antibody, AsPC-1 cells have been demonstrated to express the trk receptors but not p75^{NGFR}. The expression of the individual receptor subtypes or NTs has not been determined in this cell line (9). In addition, normal host tissue can be a source of NTs as demonstrated in clinical PDAC specimens in which expression of BDNF, NT-3, and NT4/5 has been observed in surrounding host stromal tissue (9). Similarly, normal prostatic stromal cells (but not prostatic epithelium) have been demonstrated to express NGF and BDNF, whereas prostatic carcinomas express NGF, BDNF, and NT-3 in both their stromal and epithelial components (15).

Tumor-bearing animals were administered the anti-NT antibody mixture (400 μg) or normal rabbit IgG (400 μg). In the study with PC-3 xenografts, additional experimental groups received 100 μg of anti-NT antibody mixture composed of 25 μg each of the individual neutralizing antibodies or 100 μg of normal rabbit IgG following the same dosing schedule. The most pronounced antitumor response to administration of the anti-NT antibody mixture (400 μg) was observed in the PC-3 xenografts (Fig. 1A) in which significant regression in PC-3 xenografts ($P \leq 0.05$) of 31 and 19% relative to tumors in corresponding IgG-treated control animals was achieved on days 10 and 13 of administration, respectively (Fig. 1A). Similarly, PC-3 xenografts (Fig. 1A) treated with 100 μg of anti-NT antibody mixture and the TSU-Pr1 xenografts (Fig. 1B) treated with 400 μg of antibody mixture demonstrated a significant inhibitory response to anti-NT antibody mixture treatment relative to corresponding IgG-treated control mice, beginning on day 3 [24% inhibition (PC-3); $P \leq 0.05$] and day 5 [TSU-Pr1 (38% inhibition, $P \leq 0.0001$)] and extending over the 15-day dosing period. The inhibition of PC-3 and TSU-Pr1 tumor

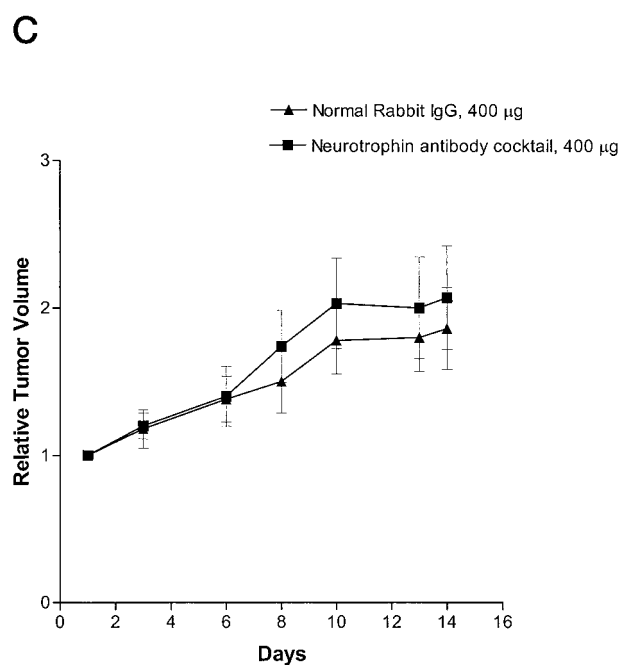
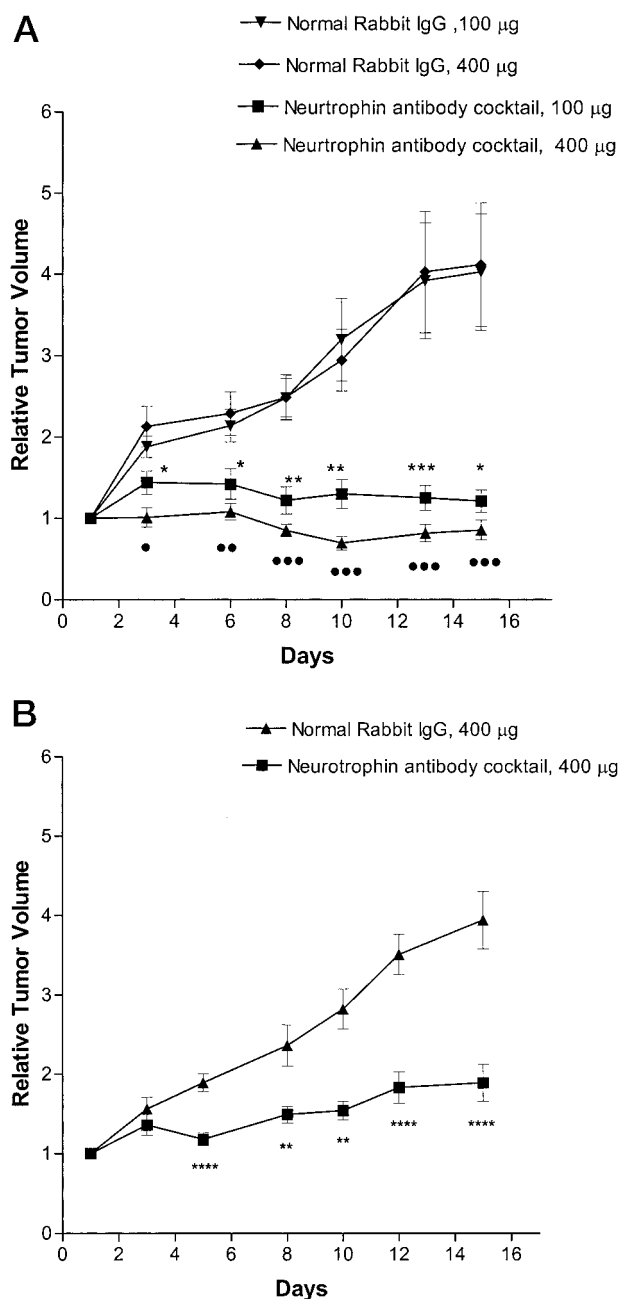


Fig. 1 Antitumor effect of NT neutralizing antibodies on human prostatic xenografts in nude mice. Athymic nude mice were injected with 5×10^6 PC-3 (A), TSU-Pr1 (B), or SK-OV-3 (C) tumor cells. When mean tumor volumes reached between 250 and 400 mm³ in size, a mixture of NT neutralizing antibodies 400 μ g (composed of 100 μ g each of anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5) or 100 μ g (composed of 25 μ g each anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5), or normal rabbit IgG (400 μ g or 100 μ g) was injected three times/week, intratumorally and peritumorally. Values are means of relative tumor volume; bars, SE. Significant differences in PC-3 relative tumor volumes (A) were observed between the groups treated with 100 μ g of NT antibody mixture and 100 μ g of normal rabbit IgG (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) and between the groups treated with 400 μ g of NT antibody cocktail and 400 μ g of normal rabbit IgG (●, $P \leq 0.05$; ●●, $P \leq 0.01$; ●●●, $P \leq 0.001$). Significant differences in TSU-Pr1 relative tumor volumes (B) were observed between the groups treated with NT antibody mixture and normal rabbit IgG (**, $P \leq 0.01$; ****, $P \leq 0.0001$). The antibodies did not affect the growth of SK-OV-3 xenografts (C). All statistical analyses were performed using the Mann-Whitney Rank Sum test.

growth by the mixture of neutralizing antibodies relative to the IgG-treated control mice confirmed that the inhibitory effects seen in these xenografts were specific to neutralizing antibody treatment and not the result of spontaneous regression of s.c. tumor xenografts (Fig. 1, A and B).

The NT anti-NT antibody mixture (400 μ g) had no effect on the growth of SK-OV-3 xenografts (Fig. 1C), although we have demonstrated that these cells express the high-affinity NGF receptor, trkA (Fig. 2). These data are in agreement with previous observations in this model using the small molecule trk kinase inhibitor, CEP-751, that also had no significant effect on the growth of SK-OV-3 xenografts (13).

Administration of the anti-NT antibody mixture (400 μ g) to nude mice bearing AsPC-1 xenografts inhibited xenograft growth significantly ($P \leq 0.05$) over the 15-day dosing period as compared with the normal rabbit IgG-treated controls (Fig. 3A). A significant response to the neutralizing antibody mixture was first observed on day 5 (35% inhibition, $P \leq 0.05$) and continued throughout the remainder of the study, with maximal inhibition of 53%. As observed with the SK-OV-3 xenografts, the CFPAC xenografts did not respond to treatment with the anti-NT antibody mixture (Fig. 3B), although they express the high affinity NGF receptor, trkA (Fig. 2). These findings are in agreement with earlier observations in which the small molecule trk kinase inhibitor, CEP-701,

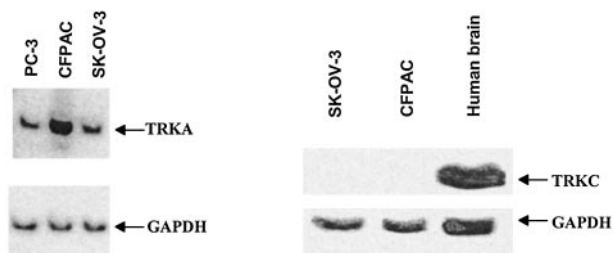


Fig. 2 RT-PCR detection of *trkA* expression in PC-3 prostatic, CFPAC pancreatic, and SK-OV-3 ovarian carcinoma tumor cell lines but not *trkC* expression in CFPAC or SK-OV-3. Total RNA was isolated from cultured human cell lines. cDNA was synthesized and subjected to PCR amplification to identify transcripts of *trkA*, *trkC*, and GAPDH.

inhibited significantly the growth and invasiveness of AsPC-1 but not CFPAC xenografts in nude mice (25).

To assess the contribution of the individual NTs to tumor growth, additional experiments were carried out using the poorly differentiated, androgen-dependent cell line, TSU-Pr1. In addition, this same prostatic/bladder carcinoma cell line was used to determine the effects of affinity-purified anti-NGF antibody on tumor growth to verify that the inhibition of tumor growth observed in the previous experiments using polyclonal anti-NT antibodies was attributable to their anti-NGF neutralizing capabilities and not attributable to another component of the antibody.

Effects of Individual Neutralizing Antibodies on the Growth of TSU-Pr1 s.c. Xenografts in Nude Mice. Tumor-bearing mice were treated with the individual neutralizing antibodies (400 μ g; composed of 100 μ g of anti-NGF, anti-BDNF, anti-NT-3, or anti-NT-4/5 plus 300 μ g of normal rabbit IgG), the anti-NT antibody mixture (400 μ g), or normal rabbit IgG (400 μ g) to determine the contribution of each individual NT to tumor growth and progression. As observed previously, treatment with the anti-NT antibody mixture inhibited the growth of TSU-Pr1 tumors significantly ($P \leq 0.05$ to $P \leq 0.01$) over the 15-day dosing period as compared with treatment with normal rabbit IgG (Fig. 4). In addition, both anti-NGF and anti-NT-3 administration alone inhibited the growth of TSU-Pr1 xenografts significantly ($P \leq 0.05$ to $P \leq 0.001$) as compared to treatment with normal rabbit IgG (Fig. 4). Anti-NGF treatment caused significant inhibition of tumor growth beginning on day 3 (62%; $P \leq 0.01$), whereas treatment with anti-NT-3 resulted in significant inhibition of xenograft growth beginning on day 6 (30%; $P \leq 0.01$). Despite a maximum inhibition of TSU-Pr1 of tumor growth of 67 and 64% with anti-NGF and anti-NT-3, respectively, neither anti-BDNF nor anti-NT-4/5 treatment alone had any effect on TSU-Pr1 tumor xenograft growth (Fig. 4).

Effects of Affinity-purified Anti-NGF on the Growth of TSU-Pr1 s.c. Xenograft Growth in Nude Mice. Tumor-bearing mice were treated with the affinity-purified anti-NGF (0.1, 1.0, and 10.0 μ g), IgG fraction of anti-NGF (100 μ g), or normal rabbit IgG (100 μ g). As observed in the previous experiment using this xenograft model, the polyclonal anti-NGF neutralizing antibody inhibited the growth of TSU-Pr1 tumors significantly ($P \leq 0.05$ to $P \leq 0.001$) over the 15-day dosing

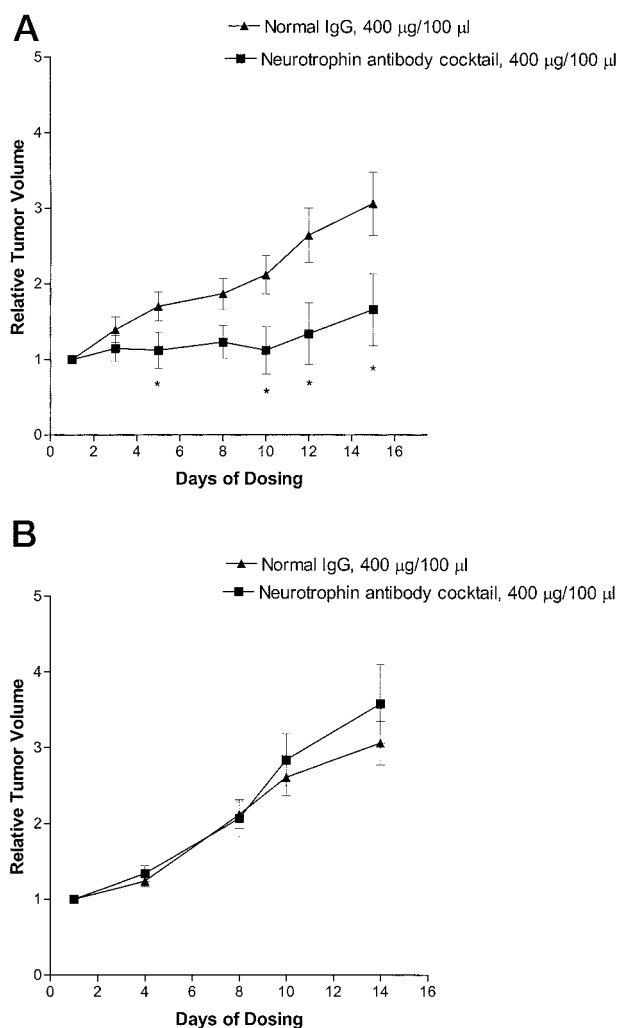


Fig. 3 Antitumor effect of NT neutralizing antibodies on human pancreatic xenografts in nude mice. Athymic nude mice were injected with 5×10^6 AsPC-1 or CFPAC tumor cells. When mean tumor volumes reached between 250 and 400 mm^3 in size, a mixture of neutralizing antibodies 400 μ g (composed of 100 μ g each of anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5) or normal rabbit IgG (400 μ g) was injected three times/week, intratumorally and peritumorally. Values are means of relative tumor volume; bars, SE. Significant differences in AsPC-1 relative tumor volumes (A) were observed between the groups treated with NT antibody mixture and normal rabbit IgG (*, $P \leq 0.05$). The antibodies did not affect the growth of CFPAC xenografts (B). All statistical analyses were performed using the Mann-Whitney Rank Sum test.

period as compared with treatment with normal rabbit IgG (Fig. 5). Treatment with affinity-purified anti-NGF (0.1, 1.0, or 10.0 μ g) also inhibited significantly ($P \leq 0.05$ to $P \leq 0.001$) the growth of TSU-Pr1 tumors in nude mice (Fig. 5). As compared with treatment with normal rabbit IgG, treatment with 0.1 or 1.0 μ g affinity-purified anti-NGF resulted in a significant inhibition of tumor growth (46 and 56%, respectively; $P \leq 0.01$) by day 10 of treatment. The most pronounced response to affinity-purified anti-NGF administration as compared with treatment with normal rabbit IgG was observed with the highest dose (10 μ g).

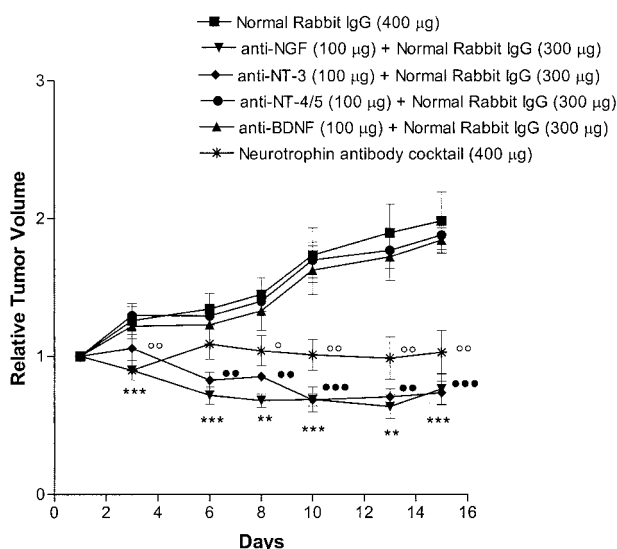


Fig. 4 Antitumor effect of individual NT neutralizing antibodies on TSU-Pr1 prostatic xenografts in nude mice. Athymic nude mice were injected with 5×10^6 tumor cells. When mean tumor volumes reached between 200 and 250 mm^3 in size, mice were injected with individual antibodies [anti-NGF (100 μg) + normal rabbit IgG (300 μg); anti-NT-3 (100 μg) + normal rabbit IgG (300 μg); anti-NT-4/5 (100 μg) + normal rabbit IgG (300 μg); anti-BDNF (100 μg) + normal rabbit IgG (300 μg); NT antibody mixture (400 μg); or normal rabbit IgG (400 μg) three times/week, intratumorally and peritumorally]. Values are means of relative tumor volume; bars, SE. Significant differences in relative tumor volumes were observed between the groups treated with anti-NGF compared with normal rabbit IgG (**, $P \leq 0.01$; ***, $P \leq 0.001$), anti-NT-3 compared with normal rabbit IgG (●●, $P \leq 0.01$; ●●●, $P \leq 0.001$), and NT antibody mixture compared with normal rabbit IgG (○, $P \leq 0.05$; ○○, $P \leq 0.01$) by Mann-Whitney Rank Sum test.

Tumors treated with 10.0 μg of affinity-purified anti-NGF were significantly smaller (30%; $P \leq 0.01$) than those treated with 0.1 μg of affinity-purified anti-NGF by day 6. Tumors treated with 1.0 μg of affinity-purified anti-NGF inhibited the growth of TSU-Pr1 tumors significantly (43%; $P \leq 0.01$) by day 12 as compared to treatment with 0.1 μg of affinity-purified anti-NGF. These data indicate that the inhibition of tumor growth observed in previous experiments using the polyclonal NT antibodies was attributable to their anti-NGF neutralizing capabilities and not some other component of the antibody preparation.

DISCUSSION

In this report, we have examined the role of the NT-trk receptor axes in the development and progression of human prostatic carcinoma and PDAC-derived xenografts in nude mice and the consequences of disrupting trk-mediated signaling in these tumor types using a mixture of NT neutralizing antibodies (anti-NGF, anti-BDNF, anti-NT-3, and anti-NT-4/5). We provide evidence that the NT-trk receptor axes are involved in the growth of TSU-Pr1 and PC-3 prostatic carcinoma and AsPC-1 PDAC xenografts. Treatment with the anti-NT antibody mixture inhibited significantly the growth of TSU-Pr1 and AsPC-1 tumor xenografts over a 15-day period and was generally well tolerated. In addition, treatment of nude mice bearing PC-3 tumors with the antibody mixture (400 μg) caused significant

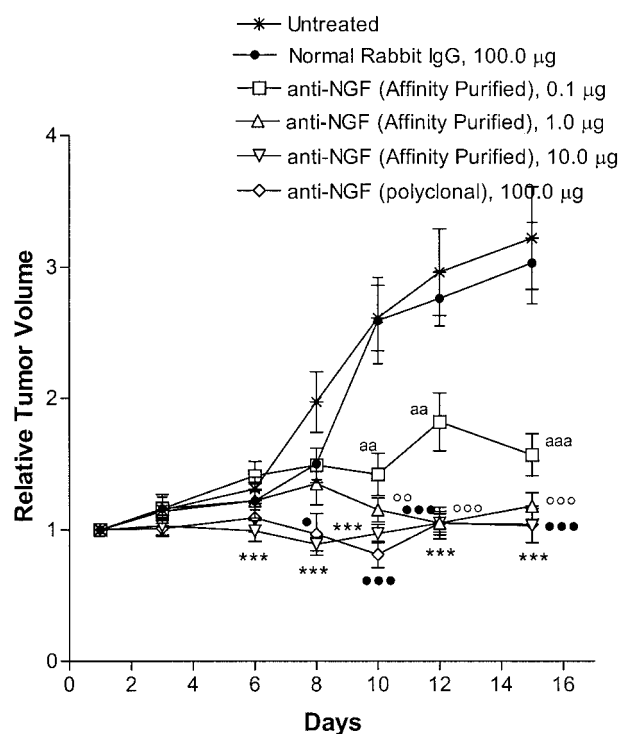


Fig. 5 Antitumor effect of affinity-purified anti-NGF neutralizing antibody on TSU-Pr1 xenografts in nude mice. Athymic nude mice were injected with 5×10^6 tumor cells. When mean tumor volumes reached between 150 and 250 mm^3 in size, mice were injected with 100 μg of anti-NGF (polyclonal); 0.01 μg anti-NGF (affinity purified); 1.0 μg anti-NGF (affinity purified); 10.0 μg anti-NGF (affinity purified) or normal rabbit IgG (100.0 μg) three times/week, intratumorally and peritumorally. Values are means of relative tumor volume; bars, SE. Significant differences in relative tumor volumes were observed between the groups treated with 0.01 μg of anti-NGF (affinity purified) compared with 100.0 μg of normal rabbit IgG (aa, $P \leq 0.01$; aaa, $P \leq 0.001$); 1.0 μg anti-NGF (affinity purified) compared with 100.0 μg of normal rabbit IgG (○○, $P \leq 0.001$; ○○○, $P \leq 0.001$); 10 μg anti-NGF (affinity purified) compared with 100.0 μg of normal rabbit IgG (***, $P \leq 0.001$); and 100 μg anti-NGF (IgG fraction) compared with 100.0 μg of normal rabbit IgG (●, $P \leq 0.05$; ●●●, $P \leq 0.001$) by Mann-Whitney Rank Sum test.

tumor regression. The mixture of NT neutralizing antibodies did not inhibit the growth of either the SK-OV-3 or CFPAC xenografts, although each cell line expressed the high-affinity NGF receptor, trkA, supporting previous data using the small molecule pan-trk inhibitors, CEP-751 and CEP-701 (13, 25). These combined data suggest that inhibition of the NT-trk receptor axes confers antitumor efficacy against selective tumor types, corroborating findings from previous studies with small molecule trk kinase inhibitors *in vitro* and *in vivo* (13, 23–25).

An important observation in the current studies was that anti-NGF or anti-NT-3, but not anti-BDNF or anti-NT-4/5, inhibited significantly TSU-Pr1 tumor growth compared with xenografts treated with the mixture of all four neutralizing antibodies. The sensitivity of TSU-Pr1 tumors to anti-NGF and anti-NT-3 is consistent with previous data demonstrating the expression of NGF and NT-3 by TSU-Pr1 cells in culture (15, 34). In addition to expressing NGF and NT-3, TSU-Pr1 cells

express trkA (13), the high-affinity NGF receptor; therefore, it is possible that the inhibition of tumor growth by anti-NGF observed in this study was attributable to blocking the NGF activation of trkA. There are conflicting reports regarding TSU-Pr1 expression of trkC, the primary high-affinity receptor for NT-3 (13, 16). Using RT-PCR methods, we were unable to detect trkC expression from TSU-Pr1 cells, although trkC was detected from control mRNA (human brain), and the integrity of the TSU-Pr1 mRNA was verified by amplification of a control gene (data not shown). In addition, TSU-Pr1 cells do not express the low-affinity NGF receptor, p75^{NGFR}, which modulates the affinity, specificity, and/or local availability of the NTs for high-affinity binding to their specific trk receptor subtypes (4, 35). On the basis of these combined data, it is possible that anti-NT-3 is most likely the inhibiting signaling mediated by NT-3 via one or more trk receptors other than trkC in TSU-Pr1 tumors, but the exact trk target has not been identified.

The lack of effect of anti-BDNF or anti-NT-4/5 neutralizing antibodies on TSU-Pr1 xenograft growth may be attributable to several possibilities. One or more of the NTs may interfere with the growth-promoting, invasive/chemotactic, or antiapoptotic activities of NGF and NT-3. Examples of antagonistic effects of NTs on cell survival have been observed for NGF and BDNF in neuroblastomas, where activation of trkA by NGF promotes neuronal differentiation, whereas activation of trkB by BDNF results in proliferation and survival of neuroblastoma cells (36). Antagonistic effects have also been observed for NT-3 and BDNF within the central nervous system, where endogenous NT-3 promotes the death of all corticospinal neurons dependent upon BDNF for survival (37). A second possible explanation for the insensitivity of TSU-Pr1 tumors to anti-BDNF and anti-NT-4/5 administration may be a reduction in ligand affinity, intratumoral antibody diffusion, and/or differential stability of anti-BDNF and anti-NT-4/5 antibodies relative to anti-NGF and anti-NT-3, because the intratumoral neutralizing activity of the antibodies was not evaluated. Finally, the lack of antitumor effect may be attributable to the absence of trkB expression in TSU-Pr1 cells, as reported by Dionne *et al.* (13) with the same source of cells used in this study.

The consequences of disrupting the NT-trk receptor axes through the use of the neutralizing antibodies on the apoptotic *versus* proliferative fraction of target tumor cells have yet to be elucidated. Support for the dependence of prostatic carcinoma cells on the NT-trk axes has been demonstrated using *in vitro* clonogenicity assays in which the survival of human prostatic carcinoma cell lines (DU-145, LNCAP, and PC-3) and the human prostatic/bladder carcinoma cell line (TSU-Pr1) was reduced by >50% by incubation with the pan-trk inhibitor, CEP-751 (15). In addition, results observed with the Dunning H hormone-sensitive tumor model in rats demonstrated a 13-fold increase in the percentage of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells 2 days after treatment with CEP-751, while causing only minimal effects on tumor proliferative fraction. Further data obtained with trk kinase inhibitors, CEP-751 and CEP-701, in the Dunning H model also showed that disruption of NT-trk axes induces death of prostate cancer cells *in vivo* independent of effects on the cell cycle (13, 38). These combined data suggest that NT-trk axes

are playing a fundamental role in the survival pathways within prostate cancer cells.

In contrast to what we have observed in prostatic carcinoma xenografts, *in vivo* disruption of NT-trk axes in PDAC xenografts have more pronounced effects on inhibiting tumor cell proliferation and less pronounced effects on the induction of apoptosis (39). In two different *in vivo* models of human PDAC xenografts (Colo 357 and AsPC-1), we have demonstrated that inhibition of NT-trk axes using the pan-trk inhibitor, CEP-701 resulted in a significant time-dependent decrease in the proliferative fraction (Ki-67 +) of tumor cells relative to vehicle-treated tumors over a 13-day time course, however, in only one of the xenograft models (AsPC-1), minimal effects on the apoptotic fraction (terminal deoxynucleotidyl transferase-mediated nick end labeling-positive) of tumor cells were observed over the same time period (39).³ Additional experiments are currently underway to elucidate the exact pathways affected by the disruption of NT-trk axes in both PDAC and prostatic carcinoma xenografts, but it is probable that specific tumor types may use trk-mediated signaling pathways differently, effecting either cell survival *versus* mitogenesis, to various degrees.

In conclusion, we have demonstrated that the intratumoral administration of a mixture of NT neutralizing antibodies inhibits the growth of human prostatic and pancreatic xenografts in nude mice. These combined results add further support for the therapeutic potential of disrupting the NT-trk axes in selected types of nonneurological human cancers, specifically prostatic and pancreatic carcinomas, which use trk signaling pathways for their proliferation and/or survival.

ACKNOWLEDGMENTS

We thank Sonya Pritchard for help with the preparation of the RT-PCR figure.

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Clin Cancer Res 2002;8:1924-1931.

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