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Bevacizumab Suppresses Neuroblastoma Progression in the Setting of Minimal Disease

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Abstract

Background—We hypothesized that vascular endothelial growth factor (VEGF) contributes to autocrine stimulation of neuroblastoma and that inhibition of its signaling pathway contributes to the anticancer activity of bevacizumab, an anti-VEGF monoclonal antibody.

Methods—For in vitro studies, two neuroblastoma cell lines, CHLA-255 and NB1691, were treated with VEGF+/-bevacizumab. For in vivo studies, disseminated neuroblastoma was established by intravenous administration of luciferase-expressing tumor cells in SCID mice prior to bevacizumab treatment.

Results—Exogenous VEGF significantly increased cell counts after 48 hours (NB1691: 58,878 ±8,279 vs. 137,500±13,108 cells, p<0.001; CHLA: 1.56×10⁶±866 vs 1.81×10⁶±2,550cells, p<0.001); the addition of bevacizumab abrogated this stimulation. In vivo, mice with disseminated disease treated twice weekly with intraperitoneal bevacizumab had a decreased tumor burden at day 14 and prolonged survival (NB1691: 50.5±2.3 vs. 42.5±1.5days, p<0.001; CHLA: 52.5±3.2 vs 42.5 ±1.4days, p=0.006). Interestingly, VEGF and basic fibroblast growth factor expression was increased in treated NB1691 tumors, likely in response to VEGF signaling inhibition.

Conclusion—Our results suggest that VEGF has a role in neuroblastoma autocrine signaling. Maintenance bevacizumab may be useful for disease suppression after maximal cytoreductive therapy. However, upregulation of pro-angiogenic factors may provide resistance to this approach, suggesting that maximal antitumor efficacy may require combination therapy.

Background

Neuroblastoma (NB) is the most common extracranial solid tumor in children, comprising approximately 7% of childhood malignancies¹ and accounting for 15% of pediatric deaths. While early stage disease can often be treated with surgery alone, advanced stage NB continues

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to have a poor prognosis despite intensive multimodality therapy. The five year survival for children with high risk NB is less than 40%². Consequently, most children with high risk disease are placed on research protocols in an effort to find new active agents or drug combinations. Because angiogenesis, or the formation of new blood vessels, appears to be a fundamental requirement for cancer progression, agents such as bevacizumab, that inhibit angiogenesis, are currently being introduced into clinical trials.

Bevacizumab is a humanized, monoclonal antibody to vascular endothelial growth factor (VEGF).³ VEGF was one of the first cytokines shown to contribute to tumor angiogenesis,⁴ and it remains one of the most important factors elaborated by tumor cells to drive new blood vessel formation.⁵ VEGF recruits endothelial cells and causes their proliferation at sites of developing blood vessels.^{4,6} VEGF also appears to sustain newly-formed, immature vessels and contributes to vascular permeability.^{7,8} As monotherapy, bevacizumab has limited efficacy in treating well-established tumors, but because of its influence on the vasculature of tumors, bevacizumab has been most effective when used in combination with other agents^{9,10} and has improved patient outcome in several clinical trials when used in combination with adjuvant cytotoxic therapy.^{11,12} VEGF binds to and mediates its effects through two tyrosine kinase receptors, VEGF Receptor 1 (VEGFR-1), also known as fms-like tyrosine kinase receptor (FLT1)¹³, and VEGF Receptor 2 (VEGFR-2), which is also referred to as kinase insert domain-containing receptor (KDR)¹⁴. VEGF also binds to the neuropilins (NRP-1 and NRP-2), which serve as co-receptors in modulating the effects of VEGF.¹⁵ These VEGF receptors and co-receptors are expressed predominantly on endothelial cells, but more recently it has been shown that tumor cells themselves can express these receptors.¹⁶ The expression of these receptors on tumor cells suggests that VEGF may play an additional role in tumor biology, through autocrine signaling, beyond its impact on tumor angiogenesis. There is some indication that VEGF expression may serve an autocrine stimulation function in some solid tumors.¹⁷ However, the role of autocrine VEGF signaling in NB progression is uncertain.

We hypothesized that tumor-elaborated VEGF contributes to autocrine stimulation of NB, and inhibition of this autocrine stimulation contributes to the anti-cancer activity of bevacizumab. Furthermore, if bevacizumab does inhibit an autocrine stimulatory effect of VEGF, then bevacizumab might also suppress minimal NB disease, slowing tumor progression. As a potential clinical application, if much of the high risk tumor burden could be removed with surgery and eradicated with chemotherapy and radiation, a monoclonal antibody to VEGF might suppress the residual disease, prolonging survival.

Methods

Cell lines

The human NB cell lines, NB1691 and CHLA-255, were provided by P. Houghton (Memphis, TN) and C. Patrick Reynolds (Los Angeles, CA), respectively. These cells were engineered to constitutively express firefly luciferase, as previously described.¹⁸ To evaluate the effect of VEGF on cell growth, cells were plated at 60,000 cells/well (NB1691) or 300,000 cells/well (CHLA-255) in 24-well tissue culture plates (Costar, Corning Incorporated, Corning, NY). After the transition to serum-free (NB-1691) or low-serum (CHLA-255) media for 24 hours, cells were washed with PBS and fresh media was added containing 10ng/ml recombinant human VEGF (rhVEGF) (PeproTech, Inc., Rocky Hill, NJ) or 10ng/ml rhVEGF and varying concentrations of bevacizumab (0.01mg/ml, 0.1mg/ml, 1mg/ml, and 10mg/ml). After 48 hours, cells were lifted with trypsin and counted with a hemocytometer.

RT-PCR for VEGF receptors and co-receptors

At approximately 80% confluence, cells were treated with 1mg/ml bevacizumab or an equal volume of media for 24 hours. Total RNA was isolated from NB cells using RNA STAT-60 (Tel-test, Inc., Friendswood, TX). cDNA was generated with reverse transcriptase of 1µg DNase-treated (Fisher Scientific, Pittsburgh, PA) RNA using SuperScript II RT (Life Technologies, Gaithersburg, MD) and then subjected to PCR amplification as previously described.¹⁹ Results are reported after 40 cycles.

Western blot analysis for ERK phosphorylation

Extracellular signal-regulated kinase (ERK) is phosphorylated following VEGF-mediated activation of VEGFR-1 and VEGFR-2. To evaluate for the activation of this receptor pathway, NB1691 and CHLA-255 cells were transitioned to serum-free media with multiple PBS and fresh serum-free media washes to remove any effects from the serum-containing media. Bevacizumab was then added at 1mg/ml to two plates and incubated at 37° C for one hour prior to receiving 0% serum-containing media or 200ng/ml rhVEGF. Three additional plates received one of the following: 0% serum-containing media, 10% serum-containing media, or rhVEGF at 200ng/ml. All plates were incubated for 3 minutes before the cells were lysed with 1 ml cold lysis buffer (50mM Tris/HCl at pH 7.4, 5mM EDTA, 150mM NaCl, 1% Triton X-100) containing complete, Mini protease inhibitor cocktail tablets (Roche Diagnostic Corporation, Indianapolis, IN), and 1mg/ml Pefabloc SC (AEBSF) protease (Roche Diagnostic Corporation, Indianapolis, IN). Lysates were iced and then clarified by microcentrifugation at 14000 r.p.m. for 10 minutes at 4° C. Proteins were added to a 12.5% SDS-PAGE gel at 50ug/sample for separation and then transferred to a nitrocellulose membrane for immunoblotting. Phosphorylated ERK was detected with a rabbit polyclonal anti-phospho-MAP kinase (Cell Signaling Technology, Danvers, MA) and appropriate secondary antibodies (GE Healthcare, Buckingham, UK). Radiographic film was used to visualize the bands.

Animal model

All murine experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. A model of disseminated neuroblastoma was established with both cell lines (NB1691 and CHLA-255) by intravenous (IV) injection of 2×10^6 luciferase-expressing cells into 4- to 6-week-old male CB-17 SCID mice (Charles' River Laboratories, Wilmington, Mass). Disease burden was measured from whole body images using a CCD camera (Xenogen, USA) by quantitating the bioluminescent signal emitted by the tumor cells following intraperitoneal administration of the substrate D-luciferin (Caliper Life Sciences, Hopkinton, MA). Mice were followed with twice-weekly bioluminescence imaging (BLI) until the first evidence of disease appeared in 75% of the mice. At this time, animals were divided into four groups of equal tumor burden based on BLI, two groups that had received NB1691 cells and two groups that had received CHLA-255 cells (n = 10 per group). One group of mice bearing NB1691 cells and one group bearing CHLA-255 cells were given twice weekly doses of intraperitoneal bevacizumab at 5mg/kg. Mice were followed with weekly BLI until death or euthanasia criteria were met. BLI signal after two weeks of treatment was divided by baseline BLI for each mouse to determine the relative tumor burden. At the time of death, intraabdominal tumor tissue was obtained for evaluation.

Growth factors

Tumor tissue obtained at harvest was snap-frozen in liquid nitrogen. Protein lysates were made by homogenizing tissue in 1ml of lysis buffer, as described.¹⁹ Fibroblast growth factor (bFGF) and VEGF were measured using a commercially available enzyme-linked immunosorbent sandwich assay (ELISA) (R&D Systems, Minneapolis, MN).

Statistical Analysis

Results were reported as mean $\pm$ SE. (sentence deleted) Kaplan-Meier curves were generated with SAS software (Version 9.1, SAS Institute, Cary, NC). A log-rank test was performed for analysis of survival in SAS software. The Sigmaplot program (SPSS, Inc, Chicago, IL) was used to graphically present all other data and analyze statistical differences in the results using an unpaired Student's t-test.

Results

In vitro analyses

By RT-PCR, both NB cell lines expressed one of the VEGF receptors or co-receptors (Figure 1). VEGFR-2 and NRP-1 were both present in NB1691. NRP-2 was present in CHLA-255. To determine whether the cell lines were responsive to VEGF stimulation, cell growth after treatment with VEGF was evaluated in vitro (Figure 2). VEGF significantly increased the cell count of both cell lines after 48 hours, compared to unstimulated controls (NB1691: $137,500 \pm 13,108$ vs. $58,878 \pm 8,279$ cells, $p < 0.001$; CHLA: $1.81 \times 10^6 \pm 2,550$ vs $1.56 \times 10^6 \pm 866$ cells, $p < 0.001$).

Western blot to determine the phosphorylation status of ERK, a downstream target of VEGFR stimulation was then evaluated to see if the administration of VEGF resulted in activation of the VEGFR signaling pathway in NB cells. Indeed, the addition of 10% fetal bovine serum or rhVEGF resulted in significant ERK phosphorylation, while cells treated with serum-free media and no VEGF had almost undetectable levels of ERK phosphorylation (Figure 3). In NB1691 cells, the addition of bevacizumab with the administration of VEGF abrogated this stimulation. While 10% serum and VEGF appeared to increase the phosphorylation ERK in CHLA-255, the effect of simultaneous bevacizumab administration was less clear. Nonetheless, consistent with increased cell proliferation after VEGF stimulation, there appears to be an increase in ERK phosphorylation downstream of VEGFR activation in both cell lines. In NB1691, this activation could clearly be ameliorated by the addition of bevacizumab.

Similarly, the simultaneous administration of 0.1mg/ml bevacizumab with VEGF caused at least a 25% drop in cell count compared to VEGF administration alone (NB1691, $p = 0.02$; CHLA, $p = 0.007$) (Figure 2). Increasing doses of bevacizumab produced further decreases in the cell count, although this decrease was less profound in the NB1691 cells (Figure 4).

Inhibition of neuroblastoma progression in vivo

Mice with disseminated disease were treated as soon after tail vein administration of tumor cells as tumor was detectable in the majority of mice by BLI. These mice were then treated with twice weekly, intraperitoneal bevacizumab or vehicle control. After two weeks of treatment, BLI was repeated to assess the relative increase in tumor burden based on a study by Dickson et al. showing that BLI signal correlated well tumor burden in NB-bearing mice¹⁸. Bevacizumab-treated mice showed a trend toward decreased tumor burden at day 14 compared to untreated mice (NB1691: 56.3 ± 13.8 vs 117.8 ± 33.5 relative tumor burden, $p = 0.112$; CHLA: 122 ± 52.4 vs 503 ± 153 relative tumor burden, $p = 0.036$) as measured by BLI (Figure 5). While treatment with bevacizumab did not ultimately prevent the progression of minimal disease, it did confer a longer survival to tumor-bearing mice. Bevacizumab-treated mice experienced a prolonged survival (NB1691: 50.5 ± 2.3 vs. 42.5 ± 1.5 days, $p < 0.001$; CHLA: 52.5 ± 3.2 vs 42.5 ± 1.4 days, $p = 0.006$), compared with untreated controls (Figure 6). Interestingly, VEGF and bFGF expression was increased in NB1691 tumors treated with bevacizumab compared to untreated tumors at harvest (VEGF 142.8 ± 34.4 vs 40.7 ± 15.3 pg/ml, $p = 0.018$; bFGF 204.4 ± 23.3 vs 99.6 ± 16.1 pg/ml, $p = 0.004$), likely in response to

downstream inhibition of VEGF signaling (Figure 7). This change was not evident in treated CHLA-255 tumors.

Discussion

VEGF blockade is primarily being used for its anti-angiogenic effects clinically, but there is increasing evidence that VEGF plays other roles in the progression of solid tumors.¹⁷ This study indicates that the progression of NB may also be affected by VEGF autocrine signaling. We found that two NB cell lines (NB1691 and CHLA-255) express VEGF receptors and co-receptors, and others have shown the presence of these receptors in other NB tumors and cell lines.^{20,21} The presence of these receptors in a VEGF-producing tumor suggests that VEGF may play an autocrine signaling role in the tumor cells. This was supported by the increased proliferation seen when cells were treated with VEGF in vitro. The addition of rhVEGF in our study also correlated with increased phosphorylation of ERK, indicating a reduction in VEGF receptor activation.

The proliferation of NB cells with VEGF stimulation presents the possibility that VEGF signaling could be a target for therapy, independent of its role in angiogenesis. This hypothesis was supported by the significant decrease in cell counts when bevacizumab was added with the VEGF. In fact, the addition of bevacizumab decreased cellular proliferation below that seen with unstimulated controls, perhaps due to the binding of endogenous VEGF from tumor cells in addition to binding of the rhVEGF added as a part of the experiment. Since NB cells are known to produce VEGF, there may be some basal autocrine stimulation of NB cells in the untreated cell cultures that is then blocked by the addition of bevacizumab in the cell cultures treated with rhVEGF and bevacizumab. In the CHLA-255 cell cultures, bevacizumab may also be binding to growth factors in the serum, and indeed, there appears to be a larger drop in cell counts below basal levels in the CHLA-255 cells (that required a minimal amount of serum to survive) than in the NB1691 cultures.

In contrast to this study, Segerstrom et al. added bevacizumab to NB cell cultures but did not find a significant difference in cell proliferation or cytotoxicity in vitro in the four cell lines tested (SK-N-AS, IMR-32, SH-SY5Y or BCE).²² The data for this analysis are not shown in the manuscript; therefore, it is difficult to make any assessment of the response or absence of response in that study in comparison to the current study. It is also not clear how the cells were maintained. If there were serum or growth factors added to the media, then there may have been too much VEGF for the bevacizumab to effectively neutralize. We maintained our cell cultures in serum-free or minimal-serum media to better control the amount of exogenous VEGF present in the culture.

In the in vivo model of disseminated disease, we chose to use the lowest dose of bevacizumab given in clinical trials (5mg/kg) and noted a decrease in tumor burden and a statistically significant increase in length of survival in tumor-bearing mice treated in the setting of minimal disease. A Children's Oncology Group phase I clinical trial of bevacizumab in pediatric patients with solid tumors was performed with doses of 5mg/kg, 10mg/kg, and 15mg/kg.²³ No dose-limiting toxicities were noted in the study. A dose of 5mg/kg produced a mean plasma concentration of 106µg/ml with a half-life of 11.9days. This is the same concentration (100 µg/ml) at which a statistically significant decrease in cell counts was seen in both cell lines in vitro. The 15mg/kg dose achieved a mean plasma concentration of 323µg/ml during the first 24-hours after treatment with a half-life of 9.6days. Mean plasma concentrations of bevacizumab remained above 100 µg/ml at every time point tested, including 14 days after treatment.

In vivo, treatment with bevacizumab also resulted in the upregulation of VEGF and bFGF. This may also support the concept of an autocrine feedback loop in which monoclonal antibody inhibition of VEGF stimulation coincided with a cellular increase in the production of VEGF. This upregulation of growth factors has been previously noted by us and others.^{19,22} More importantly, the upregulation of growth factors after treatment with a VEGF-monoclonal antibody may indicate that resistance can develop to such therapies.

Nonetheless, NB treatment with bevacizumab appears to suppress tumor growth in the setting of minimal disease. Treatment started when disease was first detectable in the mice by BLI resulted in decreased tumor burden and significantly prolonged survival. Angiogenesis inhibition may still be partially responsible for this slowed progression, but treatment was started very early when the effect of angiogenesis on tumor growth should have been minimal. Dickson et al showed that BLI detects luciferase-expressing tumors before they can even be measured with calipers or ultrasound.¹⁸ Certainly, there was minimal disease present when therapy was initiated, and treatment slowed the progression of the disease. This indicates that, in addition to its utility in affecting tumor angiogenesis, bevacizumab may play a role in the suppression of minimal NB disease. Further work is needed to establish the mechanism whereby inhibiting VEGF autocrine stimulation may suppress disease progression. Other studies have proposed possible signaling mechanisms through which this may be mediated.^{24,25} Clinically, bevacizumab may be applicable as suppressive therapy to provide a prolonged prognosis in advanced neuroblastoma after multimodality therapy has significantly debulked the tumor burden.

Our results suggest that VEGF does have a role in the autocrine signaling of NB, and that this stimulation can be targeted for anticancer therapy. Maintenance bevacizumab may be useful for suppression of minimal disease after maximal treatment for disseminated NB. However, our results also suggest that upregulation of VEGF and other pro-angiogenic factors may provide resistance to this approach, suggesting that maximal antitumor efficacy may require combination therapy.

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Reference List

1. Miller RW, Young JL Jr, Novakovic B. Childhood cancer. *Cancer* 1995;75(1 Suppl):395–405. [PubMed: 8001010]
2. Matthay KK, Villablanca JG, Seeger RC, et al. Children's Cancer Group. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. *N Engl J Med* 1999;341(16):1165–1173. [PubMed: 10519894]
3. Presta LG, Chen H, O'Connor SJ, et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997;57(20):4593–4599. [PubMed: 9377574]
4. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992;267(16):10931–10934. [PubMed: 1375931]
5. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 2005;23(5):1011–1027. [PubMed: 15585754]
6. Zachary I. Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. *Am J Physiol Cell Physiol* 2001;280(6):C1375–C1386. [PubMed: 11350732]
7. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1995;1(10):1024–1028. [PubMed: 7489357]

8. Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. *Vascul Pharmacol* 2002;39(45):225–237. [PubMed: 12747962]
9. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol* 2006;3(1):24–40. [PubMed: 16407877]
10. Dickson PV, Hamner JB, Sims TL, et al. Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. *Clin Cancer Res* 2007;13(13):3942–3950. [PubMed: 17606728]
11. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350(23):2335–2342. [PubMed: 15175435]
12. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;355(24):2542–2550. [PubMed: 17167137]
13. De Vries C, Escobedo J, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992;255:989–991. [PubMed: 1312256]
14. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci U S A* 1993;90(16):7533–7537. [PubMed: 8356051]
15. Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, Herzog Y. The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends Cardiovasc Med* 2002;12(1):13–19. [PubMed: 11796239]
16. Wey JS, Stoeltzing O, Ellis LM. Vascular endothelial growth factor receptors: expression and function in solid tumors. *Clin Adv Hematol Oncol* 2004;2(1):37–45. [PubMed: 16163158]
17. Gee MF, Tsuchida R, Eichler-Jonsson C, Das B, Baruchel S, Malkin D. Vascular endothelial growth factor acts in an autocrine manner in rhabdomyosarcoma cell lines and can be inhibited with all-trans-retinoic acid. *Oncogene* 2005;24(54):8025–8037. [PubMed: 16116481]
18. Dickson PV, Hamner B, Ng CY, et al. In vivo bioluminescence imaging for early detection and monitoring of disease progression in a murine model of neuroblastoma. *J Pediatr Surg* 2007;42(7):1172–1179. [PubMed: 17618876]
19. Davidoff AM, Ng CY, Zhang Y, et al. Careful decoy receptor titering is required to inhibit tumor angiogenesis while avoiding adversely altering VEGF bioavailability. *Mol Ther* 2005;11(2):300–310. [PubMed: 15668142]
20. Fakhari M, Pullirsch D, Abraham D, et al. Selective upregulation of vascular endothelial growth factor receptors neuropilin-1 and -2 in human neuroblastoma. *Cancer* 2002;94(1):258–263. [PubMed: 11815985]
21. Langer I, Vertongen P, Perret J, Fontaine J, Atassi G, Robberecht P. Expression of vascular endothelial growth factor (VEGF) and VEGF receptors in human neuroblastomas. *Med Pediatr Oncol* 2000;34(6):386–393. [PubMed: 10842244]
22. Segerstrom L, Fuchs D, Backman U, Holmquist K, Christofferson R, Azarbayjani F. The anti-VEGF antibody bevacizumab potently reduces the growth rate of high-risk neuroblastoma xenografts. *Pediatr Res* 2006;60(5):576–581. [PubMed: 16988184]
23. Bender J, Adamson P, Reid J, et al. Phase I trial and pharmacokinetic study of bevacizumab in pediatric patients with refractory solid tumors: a Children's Oncology Group Study. *J Clin Oncol* 2008;26(3):399–405. [PubMed: 18202416]
24. Beierle EA, Nagaram A, Dai W, Iyengar M, Chen MK. VEGF-mediated survivin expression in neuroblastoma cells. *J Surg Res* 2005;127(1):21–28. [PubMed: 15882878]
25. Beierle EA, Strande LF, Chen MK. VEGF upregulates Bcl-2 expression and is associated with decreased apoptosis in neuroblastoma cells. *J Pediatr Surg* 2002;37(3):467–471. [PubMed: 11877669]

Abbreviations

NB

Neuroblastoma

VEGF	Vascular Endothelial Growth Factor
bFGF	basic Fibroblast Growth Factor
VEGFR-1	VEGF Receptor-1
VEGFR-2	VEGF Receptor-2
NRP-1	Neuropilin Receptor-1
NRP-2	Neuropilin Receptor-2
ERK	Extracellular Signal-Regulated Kinase
BLI	Bioluminescent Imaging

Figure 1. Presence of VEGF receptors in neuroblastoma

RT-PCR detection of VEGF receptors (VEGFR-1 and VEGFR-2) and co-receptors (NRP-1 and NRP-2) in CHLA-255 and NB1691 NB tumors. HUVEC serves as a positive control, HeLa as a negative control.

Figure 2. In vitro cell proliferation with VEGF and bevacizumab

Shown are tumor cell counts in serum-free (NB-1691) or minimal-serum media (CHLA-255) after stimulation with VEGF (10ng/ml) or concomitant administration of VEGF (10ng/ml) and bevacizumab (0.1mg/ml). (* p value vs. control <0.001, ** p value vs. VEGF = 0.02, *** p value vs. VEGF = 0.007)

Figure 3. In vitro ERK phosphorylation with VEGF and bevacizumab

Western blot for phosphorylated ERK, a down-stream marker of VEGFR activation.

Figure 4. In vitro cell proliferation with increasing doses of bevacizumab

Line graph plotting the effect of increasing doses of bevacizumab (0.01mg/ml, 0.1mg/ml, 1mg/ml, and 10mg/ml) on cell proliferation of NB1691 and CHLA-255 cells.

Figure 5. Disseminated tumor burden as assessed by bioluminescent imaging after treatment with bevacizumab

Bar graph showing relative tumor burden in mice after 14 days of bevacizumab treatment compared to untreated mice, as measured by bioluminescent imaging of luciferase-expressing cells. (*p value vs. control = 0.116; **p value vs. control = 0.036)

Figure 6. Survival curves after treatment with bevacizumab

Kaplan-Meier plots for the survival of mice with disseminated NB are shown. Survival in mice treated with bevacizumab is markedly longer than untreated tumor-bearing controls (NB1691, p value <0.001; CHLA-255, p value =0.006).

Figure 7. In vivo cytokine levels after treatment with bevacizumab

These graphs represent mean ELISA results for VEGF and bFGF. After treatment with bevacizumab levels of both growth factors are upregulated in NB1691 tumors. (*p value = 0.018 vs. untreated; **p value = 0.004)