Angiogenesis, Metastasis, and the Cellular Microenvironment

A Mutated Soluble Neuropilin-2 B Domain Antagonizes Vascular Endothelial Growth Factor Bioactivity and Inhibits Tumor Progression

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Abstract
Neuropilins (NRP1 and NRP2) are coreceptors for vascular endothelial growth factor (VEGF) and mediate angiogenesis and tumor progression. VEGF binds to the NRP1 and NRP2 B domains. Previously, it was shown that mutagenesis of the soluble NRP2 B domain (MutB-NRP2) increased affinity to VEGF by 8-fold. Here, we show that MutB-NRP2 inhibited [125I]-VEGF binding to NRP1, NRP2, and VEGFR-2. It antagonized VEGF-induced VEGFR-2/NRP2 complex formation and inhibited VEGF-induced activation of AKT, a mediator of cell survival, without affecting activation of VEGFR-2. In three-dimensional embryoid bodies, a model of VEGF-induced angiogenesis, MutB-NRP2 inhibited VEGF-induced sprouting. When overexpressed in human melanoma cells, MutB-NRP2 inhibited tumor growth compared with control tumors. Avastin (bevacizumab), a monoclonal antibody to VEGF, inhibited VEGF interactions with VEGFR-2, but not with NRPs. The combination of MutB-NRP2 and Avastin resulted in an enhanced inhibition of human melanoma tumor growth compared with MutB-NRP2 treatment only or Avastin treatment only. In conclusion, these results indicate that MutB-NRP2 is a novel antagonist of VEGF bioactivity and tumor progression.

Introduction

Neuropilins (NRP) are 130 to 140 kDa single spanning transmembrane glycoproteins originally shown to be receptors for class 3 semaphorins (SEMA3; refs. 1-3). SEMA3s, such as SEMA3A and SEMA3F, are axon guidance molecules that repel axons, induce growth cone collapse, and are involved in neuronal patterning during the development of the nervous system (4). More recently, NRPs have also been shown to be coreceptors for vascular endothelial growth factor (VEGF), a potent angiogenesis factor (5), thus linking axon guidance and angiogenesis, processes characterized by branching and network formation (6).

NRP1 and NRP2 are essential regulators of vascular development. During development, NRP1 is mostly expressed in arteries, and NRP2 in veins and lymphatics (7-10). Nrp1 knockout mice (Nrp1−/−) are embryonic lethal (E12.5–E13.5) and display a completely disorganized vascular network (11). Nrp2−/− mice are viable; however, they show a severe reduction of small lymphatic vessels and capillaries (7). Nrp1/Nrp2 double-knockout mice have an even more severe phenotype. They are embryonic lethal at E8.5 and are characterized by impaired capillary formation, lack of blood vessel branching, and avascular embryos (12). In endothelial cells, NRPs function as coreceptors for VEGFR-2, leading to increased VEGF binding and enhanced migration (6). In addition, a role for NRPs in endothelial cell survival has also been reported (13, 14). However, the simultaneous expression of VEGFR-2 and NRPs on endothelial cells has made it difficult to dissect the contribution of NRPs to endothelial cell survival.

NRPs are also expressed in tumor cells and are major contributors to tumor progression and metastasis (6, 15, 16). Overexpression of NRP1 in several tumor models, including prostate carcinoma, colon carcinoma, and glioma, induced tumor angiogenesis and promoted tumor progression (17-19). Similarly, NRP2 has also been shown to promote tumor growth and metastasis in adenocarcinoma and colorectal cancer models (20, 21). In a variety of cancer patients, expression of NRPs is often upregulated and is correlated with poor prognosis (22, 23). The function of NRPs in tumor cells is still not clear. Tumor cells rarely...
express VEGFR-2; therefore, NRPs often represent the only VEGF receptors on tumor cells. As a consequence, NRPs might transduce a signal independently of VEGF in tumor cells. There are reports that the expression of NRPs correlated with tumor cell survival; for example, NRP1-mediated breast carcinoma cell survival (24). In pancreatic adenocarcinoma and colon carcinoma cells, silencing NRP2 by shRNA-NRP2 inhibited activation of AKT, implying a role for NRP2 in the activation of a cell survival pathway (20, 21).

In view of their contribution to angiogenesis and tumor progression, NRPs may represent excellent targets for novel antitumor/angiogenesis therapies. Avastin (bevacizumab), an anti-VEGF monoclonal antibody that targets VEGF interactions with VEGFR-1 and VEGFR-2, is perhaps the most effective antiangiogenesis drug available. Avastin has shown efficacy in combination with chemotherapy in the treatment of metastatic colorectal and breast cancer and non–small cell lung cancer. In addition, efficacy as a single agent has been observed in the case of renal cell carcinoma and hepatocellular carcinoma (25). Recently, it was shown that an antibody against the NRP1 B domain (anti-NRP1) had additive effects with Avastin in inhibiting tumor growth (26). Anti-NRP1 inhibited tumor angiogenesis and vascular remodeling, keeping tumor blood vessels in an immature and VEGF-responsive state (26). Anti-NRP2, an antibody against the B domain of NRP2, had no effect on angiogenesis, but strongly inhibited lymphangiogenesis and metastasis (27). However, anti-NRP2 did not affect the functionality of mature lymphatics.

Our strategy has been to generate soluble NRP-based peptides that would interact with VEGF and prevent it from binding and activating cell surface receptors. The extracellular B domain of both NRPs (B-NRP1 and B-NRP2) is essential and sufficient for VEGF binding (28-31). We have recently described a mutant of the B domain of NRP2, B-NRP2 R287E N290D (MutB-NRP2), with an affinity for VEGF of 0.74 nmol/L, almost a log higher than wild-type B-NRP2 (31). In this report, we show that MutB-NRP2 inhibited VEGF bioactivity both in vitro and in vivo. For example, MutB-NRP2 inhibited VEGFR-2 and NRP2 complex formation and VEGF-induced activation of AKT in endothelial cells. MutB-NRP2 significantly reduced VEGF-induced sprouting of murine embryonic stem cells in three-dimensional embryoid bodies, an in vitro model of vasculogenesis/angiogenesis. When overexpressed in human melanoma cells, MutB-NRP2 inhibited tumor growth significantly. Furthermore, the combination of MutB-NRP2 with Avastin was more effective than either single agent alone. Together, MutB-NRP2 seems to be a novel antagonist of VEGF bioactivity and tumor progression.

Materials and Methods

Materials

FuGENE 6 transfection reagent was from Roche Diagnostics. HEPES, NaCl, NaH2PO4, NiSO4, imidazol, bovine serum albumin, sodium orthovanadate, and heparin sodium salt from porcine intestinal mucosa were from Sigma-Aldrich, Inc. SDS sample buffer and radioimmunoprecipitation assay buffer were from Boston BioProducts, Inc. Carrier-free human VEGF and mouse VEGF were from Preprotech, Inc. VEGF was provided by the National Cancer Institute. 125I-sodium was from Perkin-Elmer Life Sciences, Inc. IODO- BEADS were from Pierce Biotechnology. NAP 5 columns were from GE Healthcare. Mouse anti-Myc antibody clone 9E10, mouse anti-NRP2 C19, and goat anti–extracellular signal-regulated kinase were from Santa Cruz Biotechnology. Rabbit anti–phospho-VEGFR-2, rabbit anti–VEGFR-2, mouse anti–phospho-AKT, rabbit anti-AKT, rabbit anti–phospho-p44/42 mitogen-activated protein kinase (MAPK), rabbit anti–phospho-p38 MAPK, and rabbit anti-p38 MAPK were from Cell Signaling Technology, Inc. Goat anti-NRP2 was from R&D Systems, Inc. Mouse anti–β-actin was from Sigma-Aldrich. ECL anti-mouse (from sheep) and anti-rabbit (from donkey) horseradish peroxidase–linked whole antibodies were from Amersham Biosciences. Peroxidase-labeled anti-goat IgG (from horse) was from Vector Laboratories, Inc. Rat anti-mouse CD31 was from BD PharMingen. Anti-rat Alexa568 and anti-rat Alexa488 were from Molecular Probes. Goat and sheep sera, and Hoechst 33342 were from Sigma-Aldrich. Complete Mini protease inhibitors were from Roche Diagnostics. Restore Western blot stripping buffer was from Thermo Scientific. Avastin (bevacizumab) was kindly provided by Dr. Mark Kieran (Dana-Farber Cancer Institute, Boston, MA). Protein G Sepharose 4 Fast Flow was from GE Healthcare. Western Lightning was from Perkin-Elmer Life Sciences, Inc. HiTrapp Chelating and HiTrapp Desalting columns were from Amersham Biosciences. SYPRO Ruby protein gel stain and Quick Start Bradford Dye Reagent were from Bio-Rad Laboratories, Inc. Zeocin was from Invitrogen. Fetal bovine serum (FBS) was from Denville Scientific, Inc. Dulbecco’s modified Eagle’s medium, 1% Glutamax, minimum essential medium, l-glutamine/penicillin G/ streptomycin sulfate mixture, CD 293 chemically defined medium and Dulbecco’s PBS were from Gibco (Invitrogen). Ham’s F-12 medium was from Cellgro Mediatech, Inc. Endothelial Basal Media-2 (EBM-2), EGM-2 Single Quots, and EGM-2 MV Single Quots were from Lonza. Leukemia inhibitory factor was from Chemicon International. Collagen type I was from Inamed Biomaterials. RNEasy Mini Kit, QiaShredder columns, and RNase inhibitor were from Qiagen. Oligo-dT primers were from Invitrogen, and deoxynucleotide triphosphates were from Fermentas. M-MLV reverse transcriptase with the corresponding reaction buffer was from United States Biochemical/Affymetrix. Sybr-Green was from Applied Biosystems.

Cell culture

Porcine aortic endothelial cells (PAEC) overexpressing NRP1 or NRP2 were established as previously described (6, 32). PAECs were grown in Ham’s F-12 medium containing 10% FBS and 1% l-glutamine/penicillin G/streptomycin sulfate. Human umbilical vein endothelial cells
(HUVECs) and human microvascular endothelial cells (HMVECs) were from Lonza, and were cultured in EBM-2 supplemented with EGM-2 Single Quots and EGM-2 MV Single Quots, respectively. HEK293 cells were from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS and l-glutamine/penicillin G/streptomycin sulfate. A375SM melanoma cells were kindly provided by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center, Houston, TX) and were maintained in minimum essential medium supplemented with 10% FBS and l-glutamine/penicillin G/streptomycin sulfate.

**Purification of MutB-NRP2**

MutB-NRP2 was generated and purified as described in ref. (31). Briefly, HEK293 cells were transfected with the pSecTag MutB-NRP2 DNA plasmid with FuGENE 6 overnight. The following day, the culture medium was replaced by serum-free CD 293 medium and conditioned medium were collected after 48 hours. The conditioned medium was centrifuged at 3,500 × g for 20 minutes, adjusted to 30 mmol/L Na2HPO4 (pH 7.4) and 150 mmol/L NaCl, filtered over a 0.22-μm filter, and applied onto a 5 mL HiTrap Chelating column loaded with NiSO4 and attached to an fast protein liquid chromatography system. After washing with 30 mmol/L Na2HPO4 (pH 7.4), 150 mmol/L NaCl, and 5 mmol/L imidazol, the proteins were eluted with 30 mmol/L Na2HPO4 (pH 7.4), 150 mmol/L NaCl, and 500 mmol/L imidazol. The fractions were analyzed by SDS-PAGE followed by SYPRO Ruby staining, and the positive fractions were subsequently desalted on a HiTrap Desalting column. The protein concentrations were determined using the Quick Start Bradford Dye Reagent.

**Iodination of VEGF and competition of binding**

VEGF was iodinated using IODO-BEADS as previously described (6). Specific activities of ~60,000 cpm/ng were obtained.

For competition binding, PAECs overexpressing NRP1, NRP2, or VEGFR-2 or HUVECs were plated on 48-well plates (50,000 per well). The following day, 125I-VEGF (5 ng/mL) was preincubated in binding buffer (Ham’s F-12, 20 mmol/L HEPES (pH 7.5), 0.5% bovine serum albumin, 1 μg/mL heparin sodium salt) with increasing concentrations of MutB-NRP2 (0–1,000 nmol/L), Avastin (0–1,000 nmol/L), or a combination of equimolar MutB-NRP2 and Avastin (0–1,000 nmol/L) at room temperature for 1 hour. Proteins were added to the cells and incubated for 1 hour at 4°C. Then, the cells were washed with cold PBS, lysed with 0.2 N NaOH, and the cell-associated radioactivity was measured in a gamma counter. The experiment was repeated in duplicates or triplicates two to three times. IC50 values were calculated from average values of independent experiments through the GraphPad software using nonlinear regression fitting parameters.

**VEGF signaling**

HUVECs or HMVECs were plated on six-well plates (400,000 cells per well). After 2 days, cells were starved overnight in EBM-2 and 0.5% FBS. The following day, cells were either left untreated or were treated with VEGF (10 ng/mL), alone or in the presence of increasing concentrations of MutB-NRP2 (0.15 and 1.5 μmol/L), for 10 minutes at 37°C. After stimulation, the cells were washed with PBS/ pervanadate and lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Complete Mini tablet) and pervanadate. Equal amounts of lysates were boiled in SDS sample buffer for 10 minutes at 95°C and analyzed by SDS-PAGE. Then, proteins were transferred onto polyvinylidene fluoride membranes and immunoblotted with phosphospecific antibodies (anti-phospho-VEGFR-2, anti-phospho-AKT, anti-phospho-p44/42 MAPK, and anti-phospho-p38 MAPK). Total proteins were detected after stripping the polyvinylidene fluoride membranes and reblotting with specific antibodies (anti-VEGFR-2, anti-AKT, anti–extracellular signal-regulated kinase, and anti-p38 MAPK).

For analysis of complex formation between VEGFR-2 and NRP2, HUVECs were either left untreated or were treated with VEGF (10 ng/mL), alone or in the presence of increasing concentrations of MutB-NRP2 (0.15 and 1.5 μmol/L), for 10 minutes at 37°C as described above. Cells were lysed and the lysates were immunoprecipitated overnight at 4°C with an anti-NRP2 antibody. Protein G slurry (50 μL) was added to the immunocomplexes, and the solution was incubated for 1 hour at 4°C. The beads were washed with radio-immunoprecipitation assay buffer, boiled in SDS sample buffer for 10 minutes at 95°C, and analyzed by SDS-PAGE followed by transfer onto polyvinylidene fluoride membranes and immunoblotting with anti-NRP2 (for loading control) and anti–VEGFR-2 antibodies.

**Embryoid bodies**

R1/SVJ 129 murine embryonic stem cells were kindly provided by Dr. Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada) and cultured as previously described (33).

At day 0, 1,200 cells were aggregated in hanging drops (20 μL) and induced to differentiate by omitting leukemia inhibitory factor as previously described (33). For sprouting assays, 4-day-old embryoid bodies were placed on top of a polymerized collagen type I gel composed of 1.5 mg/mL collagen type I in Ham’s F12 medium, 6.26 mmol/L NaOH, 20 mmol/L HEPES, 0.117% NaCO3, 1% Glutamax, and subsequently covered with a second layer of collagen type I gel to obtain a three-dimensional culture. Two hours later, VEGF at a final concentration of 30 ng/mL (0.7 mmol/L), with or without MutB-NRP2 (0.01, 0.1, or 1 μmol/L), was added. The medium was changed every 4 days. At day 12, embryoid bodies were fixed in 4% paraformaldehyde in PBS, blocked in 3% bovine serum albumin, and permeabilized in 0.2% Triton X-100 in PBS. A sequential overnight incubation with rat anti-mouse CD31 and donkey anti-rat Alexa488 followed. Hoechst 33342 was used to visualize the nuclei. Samples were inspected with a Nikon Eclipse E1000 microscope with a Nikon Eclipse DXM1200 camera (Nikon) or a LSM 510 META confocal microscope (Carl Zeiss).
For gene expression analysis, embryoid bodies were seeded in a six-well plate at day 4 in medium containing 50 ng/ml mouse VEGF. At day 9 of differentiation, RNA from embryoid bodies was extracted using the RNeasy Mini Kit and Qiashredder columns with on-column DNase digestion according to the manufacturer’s protocol. One microgram of RNA was used for reverse transcription with 1 μM Oligo-dT primers, 0.5 mMol/L deoxynucleotide triphosphates, 0.75 units RNase inhibitor, and 1 unit M-MLV reverse transcriptase in the corresponding reaction buffer for 1 hour at 37°C followed by enzyme inactivation (94°C, 10 minutes). Quantitative real-time PCR was carried out with SYBR green, 0.25 μMol/L forward and reverse primer, and 0.4 μL cDNA per reaction, with mouse hprt serving as internal control. The primer sequences are shown in Supplementary Table S1. All reactions were done in duplicate on an MX3005 instrument (Stratagene). For gene expression analysis, relative expression values were calculated according to the following formula: relative expression gene = 2^-(Ct gene - Ct internal reference). Relative expression values were normalized to the reference value (day 4 embryoid bodies).

Generation of stable clones of A375SM cells overexpressing MutB-NRP2

A375SM cells were transfected with pSecTag mock or pSecTag MutB-NRP2 DNA plasmid with FuGene 6. After 48 hours, Zeocin was added to select positive pools; then, single clones were isolated (mock: E3 and F2; MutB-NRP2 overexpressing: C9, D3, E9, and F5).

Animal tumor studies

Eight-week-old female nude mice were purchased from Massachusetts General Hospital (Boston, MA). The mice were maintained under specific pathogen-free conditions in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The care and treatment of experimental animals were in accordance with Institutional Animal Care and Use Committee guidelines at Children’s Hospital Boston. A375SM cells (1 × 10⁶ in 100 μL HBSS), mock (clones E3 and F2) or overexpressing MutBNRP2 (clones D3 and F5), were injected subcutaneously (s.c.) into the right dorsal flank of the mice (n = 8 per group). The experiment was repeated twice with consistent results.

For the combination of Avastin with MutB-NRP2, two mock clones (E3 and F2) and two MutBNRP2-overexpressing clones (D3 and F5) were selected. A375SM cells (1 × 10⁶), mock or overexpressing MutB-NRP2, were injected s.c. into the right dorsal flank of the mice (n = 16 per group). After tumors were established (~1 week), each group was divided into two subgroups and intraperitoneal (i.p.) treatment started—either with PBS (100 μL); control group; n = 8) or with Avastin (5 mg/kg/twice week in 100 μL PBS; n = 8). Tumors were measured twice per week with a caliper. Tumor volumes were calculated using the formula width² × length × 0.52. Mice were weighed twice per week to monitor eventual weight loss. After 7 weeks, the mice were sacrificed, and tumors were excised, weighed, and prepared for histology.

Immunofluorescent CD31 staining and quantification of blood vessels

Frozen sections (8-μm thick) were fixed in acetone (5 minutes) followed by ace tone/chloroform (1:1, v/v; 5 minutes) and acetone (5 minutes). Sections were blocked in goat (2%) and sheep (3%) serum for 30 minutes before staining. CD31 was detected using a rat anti-mouse CD31 antibody followed by incubation with anti-rat Alexa 568 antibody. Nuclei were stained with 4,6-diamidino-2-phenyindole.

After staining, nonnecrotic areas (as determined by 4,6-diamidino-2-phenyindole staining) were imaged at 20× magnification using a Nikon E600 fluorescent microscope. Sections from three tumors per clone were analyzed. Depending on the size of the original tumor, a range of 5 to 20 images per section were acquired, covering the entire cross-section. Vessel counts/region of interest, average vessel area, and total vessel area/region of interest were obtained using IPLab software (Scanalytics, Inc.).

Statistical analysis

Data were analyzed using the GraphPad Prism software. Unpaired two-tailed Student’s t test or two-way ANOVA analysis were done.

Results

Soluble MutB-NRP2 inhibits VEGF receptor binding, VEGFR-2/NRP2 complex formation, and VEGF-induced AKT activation

We have previously generated a soluble mutant of the B domain of NRP2 (MutB-NRP2) by site-directed mutagenesis. The mutant had an 8-fold increased affinity for VEGF compared with wild-type B-NRP2 (31). On the other hand, a reduced interaction with VEGF-C was observed (not shown). The inhibition of VEGF binding to NRP1, NRP2, and VEGFR-2 by MutB-NRP2 was tested on PAECs engineered to overexpress each receptor individually (Fig. 1A). MutB-NRP2 inhibited binding of 125I-VEGF to NRP1, NRP2, and VEGFR-2, in a dose-dependent manner, with IC₅₀ values of 75.19, 46.4, and 47.79 nmol/L, respectively (Fig. 1B; Table 1).

In the absence of VEGF, NRP2 and VEGFR-2 formed a complex in HUVECs (Fig. 1C, lane 1). However, upon addition of VEGF (10 ng/mL) or with VEGF in the presence of increasing amounts of MutB-NRP2. MutB-NRP2 did not inhibit VEGF-induced phosphorylation of VEGFR-2 (Fig. 1D) or the activation of downstream proteins such as p44/42 MAPK and p38 MAPK (not shown). However, AKT phosphorylation was inhibited by ~50% by MutB-NRP2 (Fig. 1E). Similar results were observed in another endothelial cell line, HMVEC (Supplementary Fig. S1).
MutB-NRP2 inhibits VEGF-induced sprouting of embryoid bodies

NRPs are key regulators of endothelial cell organization and branching during sprouting angiogenesis (11, 12). Embryoid bodies derived from differentiating murine embryonic stem cells have been used to analyze the mechanisms of VEGF-driven sprouting angiogenesis (34). NRPs and VEGFR-2 expression levels were analyzed by quantitative real-time PCR. After 9 days of differentiation, NRP1 (100-fold), NRP2 (40-fold), and, to a lesser extent, VEGFR-2 (10-fold) mRNA levels were significantly upregulated during VEGF-induced differentiation (Fig. 2A). The mRNA levels are relative to day 4 when VEGF was added. MutB-NRP2 was tested for the ability to inhibit the sprouting process (Fig. 2B). Embryoid bodies were induced to aggregate with the “hanging drop” method. At day 4, the embryoid bodies were placed in a collagen gel in the absence (control) or presence of VEGF alone or with increasing concentrations of MutB-NRP2. At day 9, embryoid bodies were fixed and stained for CD31 expression to visualize endothelial cell sprouting. MutB-NRP2 decreased embryoid body sprout length in a dose-dependent manner.

Table 1. IC50 values of Fig. 1B data determined by GraphPad Prism using nonlinear regression fit parameters

<table>
<thead>
<tr>
<th></th>
<th>PAEC NRP1</th>
<th>PAEC NRP2</th>
<th>PAEC VEGFR-2</th>
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<tbody>
<tr>
<td>Best-fit values</td>
<td>Log IC50 1.876 ± 0.1551</td>
<td>1.667 ± 0.1489</td>
<td>1.679 ± 0.1489</td>
</tr>
<tr>
<td>IC50 (nmol/L)</td>
<td>75.19</td>
<td>46.41</td>
<td>47.79</td>
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MutB-NRP2 (1 μmol/L) significantly reduced the length of the sprouts (48% inhibition; \( P = 0.0123 \)) compared with embryoid bodies treated with VEGF alone (Fig. 2B and C). There was a trend toward a reduction of number of sprouts per embryoid bodies upon addition of MutB-NRP2; however, this was not significant (not shown). The average sprout area and the number of branching points were not affected by MutB-NRP2 (not shown). It was concluded that VEGF-NRP2 interactions contributed to endothelial cell tube sprout lengthening and that blocking these interactions by MutB-NRP2 inhibited this process.

**Overexpression of MutB-NRP2 in melanoma cells inhibits tumor growth**

The studies on embryoid bodies indicated that MutB-NRP2 was a potential angiogenesis inhibitor and, by analogy, possibly an inhibitor of tumor angiogenesis as well. The ability of MutB-NRP2 to inhibit *in vivo* tumor angiogenesis and tumor growth was tested in a human melanoma xenograft model (A375SM). A375SM cells express NRP2 but virtually no NRP1 and no VEGFR-2 (Fig. 1A). A375SM melanoma cells were stably transfected with MutB-NRP2, and single clones were generated (Fig. 3A). Overexpression of MutB-NRP2 in A375SM did not affect the levels of endogenous NRP2 (Fig. 3A). A375SM melanoma cells overexpressing MutB-NRP2 did not have any differences in proliferation rates compared with mock-transfected cells (not shown). Two mock clones and two MutB-NRP2–overexpressing clones were chosen for tumor studies (\( n = 8 \) per group). Tumor cells were injected s.c. into nude mice as described in Materials and Methods. Tumor growth was followed for 7 weeks (Fig. 3B). The average tumor growth of the two mock clones and of the two clones overexpressing MutB-NRP2 is shown in Fig. 3B. A detailed analysis of the unaveraged individual clones is shown in Supplementary Fig. S2. Overexpression of MutB-NRP2 resulted in a ∼53% inhibition of tumor growth compared with mock (\( P < 0.001 \); Fig. 3B). After 7 weeks, the mice were sacrificed, and the
tumors were excised (Fig. 3C) and weighed (Fig. 3D). MutB-NRP2-overexpressing tumors displayed significantly lower weights (~50% weight reduction) compared with the mock control tumors ($P = 0.0163$). Snap-frozen tumor sections were stained for CD31 to analyze microvessel density (MVD). Nonnecrotic areas of three tumors per clone were imaged. However, there were no differences between mock tumors and tumors overexpressing MutB-NRP2 in average vessel counts/field, average vessel area, or total vessel area/field (Supplementary Fig. S2B).

**VEGF binding to NRPs is not inhibited efficiently by Avastin**

VEGF is a modular protein consisting of eight exons. VEGF binds VEGFR-2 through exon 4-encoded residues, whereas it interacts with NRP1 and NRP2 through exon 7/8-encoded residues. Avastin (bevacizumab), a monoclonal anti-VEGF antibody that targets exons 3/4-encoded residues, efficiently inhibited $^{125}$I-VEGF interaction with VEGFR-2 overexpressed in PAECs, with an IC$_{50}$ of 0.663 nmol/L (Fig. 4A and Table 2). However, Avastin did not inhibit $^{125}$I-VEGF binding to NRP1 (IC$_{50}$ 2.161 μmol/L; Table 2) or to NRP2 (IC$_{50}$ 47.085 μmol/L; Table 2). At the highest Avastin concentration (1,000 nmol/L, log [inhibitor] of 3.0), VEGF binding to VEGFR-2 was inhibited by more than 90%. On the other hand, at the highest concentration (1,000 nmol/L), Avastin inhibited only ~30% and 10% of $^{125}$I-VEGF binding to PAEC NRP1 and NRP2, respectively. These results suggest there are two VEGF pathways, Avastin sensitive (mediated by VEGFR-2) and Avastin resistant (mediated by NRPs).

VEGF binding experiments were carried out in HUVECs (Fig. 4B and Table 3), which express all three receptors: NRP1, NRP2, and VEGFR-2 (shown in Fig. 1A). Avastin...
inhibited $^{125}$I-VEGF binding to HUVECs poorly (IC$_{50}$ 1.8 μmol/L; Table 3). Only 30% of the total $^{125}$I-VEGF was displaced from the cell surface at the highest Avastin concentration (1,000 nmol/L; Fig. 4B). On the other hand, MutB-NRP2 displaced $^{125}$I-VEGF from the surface of HUVECs by 90%, with a 10-fold higher efficacy than Avastin (IC$_{50}$ 166.7 nmol/L for MutB-NRP2 versus IC$_{50}$ 1.8 μmol/L for Avastin, respectively; Fig. 4B and Table 3). When both Avastin and MutB-NRP2 were used in combination at equimolar concentrations to antagonize $^{125}$I-VEGF binding to HUVECs, an additive effect was observed with an IC$_{50}$ of 48.48 nmol/L (Table 3). The combination of Avastin and Mut-B-NRP2 antagonized binding of $^{125}$I-VEGF to HUVECs by 97% (Fig. 4B). These results suggested that targeting VEGF with a combination of Avastin, which inhibits VEGF–VEGFR-2 interactions, and Mut-B-NRP2, which targets VEGF-NRP1/2 interactions, might be an innovative strategy to inhibit virtually all VEGF bioactivity.

The combination of Avastin and MutB-NRP2 is more effective than either one alone in inhibiting tumor growth

It has been shown previously that the combination of Avastin with an anti-NRP1B antibody had additive effects on the inhibition of tumor growth (26). Consistent with these results, the combination of MutB-NRP2 and Avastin showed additive effects on inhibiting VEGF binding to HUVECs (Fig. 4B). Accordingly, whether there were additive effects of Avastin and MutB-NRP2 in vivo was tested in a xenograft model of A375SM melanoma (Fig. 5). Two mock clones and two clones overexpressing MutB-NRP2 were analyzed in vivo. A375SM cells (1 × 10$^6$) were injected s.c. into nude mice ($n$ = 16 per group). When tumors were palpable, each group of mice was divided into two subgroups that received injections i.p. of either PBS (controls) or Avastin (5 mg/kg, twice per week). Thus, the groups (each consisting of two clones) included: (a) mock, (b) mock treated with Avastin (Mock + Avastin), (c) A375SM overexpressing MutB-NRP2 (MutB-NRP2), and (d) A375SM overexpressing MutB-NRP2 treated with Avastin (MutB-NRP2 + Avastin). The average tumor growth of the four groups over 7 weeks was measured (Fig. 5A). A detailed analysis of the growth of unaveraged individual clones is shown in Supplementary Fig. S3. Avastin inhibited tumor growth by $\sim$55% compared with mock ($P$ < 0.001; Fig. 5A). A similar extent of inhibition (47%) was observed with MutB-NRP2 ($P$ < 0.001; Fig. 5A). Treatment with the combination of Avastin and Mut-B-NRP2 resulted in a 74% inhibition of tumor growth compared with mock. This combination was more efficient than Mut-B-NRP2 alone ($P$ < 0.01) or Avastin alone (Fig. 5A). After 7 weeks of treatment, mice were sacrificed and tumors were excised. Representative tumors are shown in Fig. 5B. Tumors overexpressing MutB-NRP2 and treated with Avastin had an average weight lower than either the MutB-NRP2–overexpressing
tumors ($P = 0.0035$) or Avastin-treated tumors ($P = 0.0069$; Fig. 5C).

Frozen tumor sections were stained for CD31. As expected, Avastin significantly reduced the average number of vessels per field ($P = 0.0212$) compared with mock (not shown). However, overexpression of MutB-NRP2 had no effect on MVD (not shown), and neither did the combination of Avastin with MutB-NRP2 (not shown) as compared with Avastin alone.

**Discussion**

NRP1 and NRP2 contribute to tumor progression and metastasis (15, 16, 26, 27). These two receptors bind VEGF on endothelial cells and tumor cells, thus implicating NRPs as regulators of tumor angiogenesis and tumor progression (6). NRPs also regulate endothelial cell and tumor cell survival in response to VEGF (20, 21, 24). Thus, targeting NRPs is a promising strategy for the development of efficient antiangiogenesis or tumor therapies. For example, anti-NRP1 antibodies in combination with Avastin inhibited tumor growth in preclinical models (26). Recently, it was shown that a peptide targeting the transmembrane domain of NRP1 inhibited glioma growth in vivo (35). Other NRP antagonists include peptide mimetics of VEGF exons that bind NRPs (VEGF exons 7 and 8). These peptides include EG3287, Tuftsin and its analogue TKPPR, and A7R (36-39). Class-3 semaphorins, the other ligands for NRPs, competed with VEGF-NRP interactions (31, 40). However, crystallographic evidence indicated that the VEGF and SEMA3A binding sites were not in proximity; thus, the basis for the competition is not clear (41). Nevertheless, SEMA3F, acting through NRP2, inhibited tumor cell and endothelial cell migration, adhesion, and invasion (42, 43). Furthermore, SEMA3F was a potent inhibitor of angiogenesis and metastasis in vivo (32).

Our goal was to develop novel peptides that blocked VEGF-NRP2 but did not block SEMA3F-NRP2 interactions that inhibit tumor progression. VEGF binds to the extracellular NRP2 B domain (B-NRP2), whereas SEMA3F interacts poorly with the B-NRP2 and needs contact with both the A and B extracellular domains of NRP2 for optimal interaction (31). We previously mutated the NRP2 B domain (MutB-NRP2) to increase VEGF affinity (31). MutB-NRP2 inhibited VEGF interactions with NRP1, NRP2, and VEGFR-2. On the other hand, MutB-NRP2 did not affect SEMA3F-NRP2 interactions (31), thus maintaining the angiogenic and antitumortastic activities of SEMA3F. NRPs formed complexes with VEGFR-2 (14, 26, 27, 44). Complex formation could either be VEGF induced (26, 44) or VEGF independent (14, 27). In our study, VEGF induced VEGFR-2/NRP2 complex formation; however, the addition of MutB-NRP2 inhibited this VEGF-induced complex by ~70%. On the other hand, no inhibitory effect of MutB-NRP2 was observed on the VEGF-induced phosphorylation of VEGFR-2 and downstream p44/42. These results were consistent, however, with previous reports that, despite strong inhibition of VEGFR-2/NRP1 complex by
anti-NRP1<sup>B</sup>, the antibody only slightly inhibited phosphorylation of VEGFR-2 and did not affect p44/42 MAPK activation (26). Similarly, anti-NRP2<sup>B</sup> strongly antagonized VEGFR-2/NRP2 and VEGFR-3/NRP2 complex formation, but the antibody inhibited phosphorylation of VEGFR-2 only mildly (27). p38 MAPK is a downstream effector of the activation of VEGFR-2/NRP1 complex by VEGF (45). MutB-NRP2 did not inhibit the activation of p38 MAPK, whereas anti-NRP1<sup>B</sup> showed only a slight inhibition of VEGF-induced p38 MAPK activation (26).

Unlike the ineffectiveness of MutB-NRP2 in inactivating VEGF-induced VEGFR-2, p44/42 MAPK, and p38 MAPK phosphorylation, MutB-NRP2 inhibited VEGF-induced AKT phosphorylation in endothelial cells by 50%. AKT/PKB is a major regulator of cell growth, proliferation, and survival (46, 47). AKT/PKB is activated downstream of phosphoinositide 3-kinase and it is often deregulated in several human cancers (46, 47). One possibility is that, in endothelial cells, NRPs induce survival by amplifying VEGF-2 signaling, acting as coreceptors of VEGFR-2 (14). However, it has also been shown that NRP1 can mediate activation of survival independently of VEGF-2. This survival pathway involves neuropilin interacting protein (NIP/GIPC), which binds to the SEA (Ser-Glu-Ala) sequence of NRP1 and NRP2, and its consequent activation of phosphoinositide 3-kinase and AKT (13). Overexpression of NRP1 in breast cancer cells induced AKT activation and increased cell survival (24). Silencing of NRP2 in pancreatic adenocarcinoma and colon carcinoma cells resulted in inhibition of AKT activation, further suggesting that NRPs play a role in the survival of tumor cells (20, 21). We hypothesize that MutB-NRP2 inhibits AKT activation by preventing VEGF binding to NRPs on the cell surface and consequently inhibiting VEGF-induced activation of NRPs. It is also possible that by blocking VEGF-2/NRP2 complex formation, MutB-NRP2 affects the downstream activation of AKT.

NRPs regulate endothelial cell organization during sprouting angiogenesis (11, 12). Effects of MutB-NRP2 on angiogenic sprouting were analyzed in differentiating embryoid bodies, an in vitro model of vasculogenesis/angiogenesis. Embryoid body sprouting was induced by VEGF. In the presence of Mut-B-NRP2, there was a 50% reduction in sprout length, suggesting that NRPs were involved in the sprouting process. The inhibition of embryoid body sprouting suggested that MutB-NRP2 might also possibly inhibit tumor angiogenesis in vitro. Accordingly, human melanoma cells overexpressing MutB-NRP2 or mock were grown in immunocompromised mice. Tumors overexpressing Mut-B-NRP2 were significantly smaller (~50% of volume reduction) than the mock tumors. Unexpectedly, there were no differences in MVD, as measured by vessel counts per field, between mock tumors and tumors overexpressing Mut-B-NRP2. However, it has been previously shown that antiangiogenic therapies do not always result in reduction of MVD, and a lack of change in MVD does not necessarily reflect a lack of efficacy in an antiangiogenic drug (48). It could be that inhibiting angiogenesis lowers blood vessel counts but that the loss of blood vessels is concomitant with tumor cell death so that the resulting MVD is not altered (48). Besides effects on tumor angiogenesis, MutB-NRP2 might have a direct effect on inhibiting tumor cell survival; namely, MutB-NRP2 might directly inhibit binding of VEGF to NRP2 on tumor cells, thereby inhibiting tumor cell survival. This mechanism would be consistent with previous reports showing a correlation between NRP expression and tumor cell survival (20, 21, 24).

Avastin (bevacizumab), an anti-VEGF antibody, is a very effective antiangiogenesis drug. Recent preclinical studies have shown that combining Avastin treatment with anti-NRP1<sup>B</sup> treatment resulted in additive effects in inhibition of tumor growth (26). In our study, Avastin inhibited VEGF interactions with VEGFR-2, but, surprisingly, Avastin did not inhibit VEGF (exons 7/8) interactions with NRPs. MutB-NRP2 had an additive effect with Avastin on the inhibition of 125I-VEGF binding to endothelial cells in vitro. Furthermore, an Avastin-MutB-NRP2 drug combination inhibited tumor progression better than either one alone. Tumors overexpressing Mut-B-NRP2 treated with Avastin grew more slowly and weighed substantially less than Avastin-only treated tumors. The combination therapy resulted in significantly smaller tumors compared with the Mut-B-NRP2-overexpressing tumors. These results suggest that targeting both pathways simultaneously will improve anti-VEGF therapy.

In summary, the extracellular B domain of NRP2 mutated to obtain higher affinity for VEGF (MutB-NRP2) is a potent VEGF antagonist that inhibits VEGF-induced VEGFR-2/NRP2 complex formation, AKT activation, angiogenic sprouting in vitro, and tumor progression. A combination of MutB-NRP2 overexpression and Avastin delivery inhibits tumor growth more efficiently than either treatment alone. We conclude that MutB-NRP2 is a novel inhibitor of tumor progression that complements Avastin inhibitory activity.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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