The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth

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The soluble extracellular domain of EphB4 (sEphB4) was designed as an antagonist of EphB4/EphrinB2 signaling. sEphB4 blocks activation of EphB4 and EphrinB2; suppresses endothelial cell migration, adhesion, and tube formation in vitro; and inhibits the angiogenic effects of various growth factors (VEGF and bFGF) in vivo. sEphB4 also inhibits tumor growth in murine tumor xenograft models. sEphB4 is thus a therapeutic candidate for vascular proliferative diseases and cancer. (Blood. 2006;107:2330-2338) © 2006 by The American Society of Hematology

Introduction

Differentiation of mesodermal cells to angioblasts occurs with simultaneous commitment to either arterial or venous lineage. Angioblasts spontaneously aggregate, proliferate, and differentiate to form endothelial tubes of each lineage. Independently developing arterial and venous vascular networks eventually join to form the original cardiovascular loop in the process of vasculogenesis.1-3 Sprouting of new vessels from this primary complex, or angiogenesis, is mediated by growth factors that induce endothelial cell (EC) proliferation, migration, and assembly, followed closely by the recruitment of perivascular cells, including smooth muscle cells, and remodeling of the extracellular matrix.4,5 A number of EC-specific receptor tyrosine kinases have been identified that play important roles in the early development of blood vessels and formation of the cardiovascular system, and include VEGF receptors and Tie-1 and Tie-2 receptors.5-11 More recently, Eph receptors and their ligands have been shown to play a critical role in the development and maturation of the cardiovascular system.11-13

The Ephs and Ephrins together comprise the largest of the receptor tyrosine kinase subfamilies (with 14 receptors and 8 ligands) and are subdivided into EphA and EphB categories based on sequence homologies and binding properties to Ephrin ligands. EphA receptors bind to glycosylphosphatidylinositol (GPI)-anchored Ephrin ligands (EphrinA subfamily), whereas EphB receptors bind Ephrin ligands that contain transmembrane and cytoplasmic domains (EphrinB subfamily).14 The extracellular domain of Eph receptors consists of a ligand-binding (globular or G) and a cysteine-rich (C) domain followed by 2 fibronectin III-like repeats (F1 and F2). The intracellular domain contains an autoinhibitory tyrosine in the juxtamembrane region, followed by a kinase domain, sterile α and PDZ-binding motifs.15,16

Eph receptor tyrosine kinases and their Ephrin ligands regulate a diverse array of cellular functions such as cell migration, repulsion, and adhesion, but lack effects on cell proliferation.9,17-21 These functions are dependent on bidirectional signals between cells expressing receptors and cells expressing ligands, which, for uniformity of communication, are termed “forward” and “reverse” signaling, respectively.6,8,11,21-25 EphrinB2 is specifically expressed in arterial angioblasts and endothelial and perivascular mesenchymal cells, whereas EphB4 is expressed in ECs belonging to the venous lineage only.12,26-28 EphrinB2 is the sole ligand for EphB4, although EphrinB2 can induce and be induced by several EphB receptor members.29,30 Targeted disruption of either EphB4 or EphrinB2 results in early lethality in the developing embryo as a result of arrest in angiogenesis but not vasculogenesis.12,26,28,31 However, recent studies indicate that inactivation of reverse signaling (mouse EphrinB2 knock-out and knock-in of C-terminal truncated EphrinB2) allows angiogenesis to occur in an orderly, sequenced manner.32

On binding, the receptor and ligand on adjacent cells undergo dimerization and clustering, which are required for further activation. This feature led us to test whether the monomeric form of the extracellular domain of EphB4 will function as an antagonist of EphB4-EphrinB2 interaction. Specifically, we hypothesized that the soluble extracellular domain of EphB4 (sEphB4) will not itself activate EphrinB2 and also block phosphorylation of EphB4 and EphrinB2 on cells when stimulated by clustered EphrinB2 or EphB4, respectively. Pursuant to these biochemical effects, s EphB4 was predicted to modulate the migration of ECs and maturation to form tubelike structures in response to growth factor stimuli in biologic assays in vitro and in vivo. Lastly, such effects of sEphB4 would retard angiogenesis as a whole and tumor growth, in particular. Recombinant soluble extracellular domain of EphB4, containing the globular ligand-binding subdomain, indeed fulfills these expectations and is thus a candidate for formal investigation...
in human diseases in which vascular proliferation has a significant contribution.

Materials and methods

Expression constructs

Two soluble proteins encoding the entire extracellular portion of EphB4 (sEphB4) and its N-terminus truncated analog (CF2) were generated. To produce the entire extracellular domain of EphB4 (soluble EphB4 [sEphB4]), the encoding fragment was amplified from full-length human EphB4 cDNA by polymerase chain reaction (PCR) using TACTAGTCCGCATGGAGTGGTTGATGATGCTGCTCCGCCAGCTCCGGGCTGCTGCTGCGGTG as a direct primer and TGGCGGCGGATTAATGGT-GATGGTGATGATGCTGCTCCGCCAGCTCCGGGCTGCTGCTGCGGTG as a reverse primer. To generate the CF2, N-terminal truncated analog of sEphB4 in which the G-region is deleted (CF2), a reverse oligo primer. To generate the CF2, N-terminal truncated analog of sEphB4 in which the G-region is deleted (CF2), two overlapping fragments encoding for sEphB4 were obtained separately by PCR amplification. For this purpose, 2 pairs of oligo primers were used: TACTAGTCCGCATGGAGTGGTTGATGATGCTGCTCCGCCAGCTCCGGGCTGCTGCTGCGGTG with Coomassie blue, Western blotting, and UV spectroscopy. Purified proteins were dialyzed against 20 mM Tris-HCl, 0.15 M NaCl, pH 8, and stored at −70°C.

Ligand-binding assay

To test EphrinB2-binding properties of the expressed and purified proteins, 10 μL Ni-NTA-agarose beads (Qiagen) were incubated in microcentrifuge tubes with 1 to 50 ng His-tagged proteins diluted in 0.2 mL binding buffer (20 mM Tris-HCl, 0.15 M NaCl, 0.1% bovine serum albumin, pH 8). After incubation for 30 minutes on a shaking platform, beads were washed twice with 1.4 mL binding buffer, followed by application of 200 ng EphrinB2-AP fusion protein in a final volume of 0.2 mL. Binding was performed for 45 minutes on a shaking platform and tubes were centrifuged and washed twice with 1.4 mL binding buffer. The amount of precipitated AP was measured colorimetrically at 405 nm after application of p-nitrophenyl phosphate. The experiment was repeated 3 times.

Immunoprecipitation and cell-based EphB4 and EphrinB2 tyrosine kinase assay

Typically, human umbilical vein endothelial cells (HUVECs) were grown in 60-mm dishes until 100% confluence and were treated with either clustered EphrinB2-Fc at 1 μg/mL in RPMI for 10 minutes to activate EphB4 receptor or Fc fragment alone as a negative control. To study the effect of different derivatives of soluble EphB4 proteins on EphB4 receptor activation, sEphB4 or CF2 was premixed with EphrinB2-Fc at a 1:5 molar ratio and incubated for 20 minutes followed by application to cells for 10 minutes.

After stimulation, media was aspirated and cells were immediately harvested with protein extraction buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM sodium vanadate, pH 7.8. Protein extracts were clarified by centrifugation at 18 000 g for 20 minutes at 4°C. Clarified protein samples were incubated overnight with protein A-agarose precoated with mAb138. The precipitated immune complexes were washed twice with the same extraction buffer containing 0.1% Triton X-100, solubilized in 1 × SDS-PAGE loading buffer and separated on 10% SDS-PAGE. For EphB4 receptor activation studies, electrophoretic membrane was probed with anti-p-Tyr-specific antibody 4G10 at 1:1000 dilution followed by application of protein G-HRP conjugate (Sigma, St Louis, MO) at 1:5000 dilution.

For EphrinB2 phosphorylation assay, 293T cells were transiently cotransfected with EphrinB2-Myc tag and EphB4 plasmid DNAs. Transfected cells were either treated or nontreated with sEphB4 at a final concentration of 5 μg/mL for 24 hours. Cell lysates were prepared as described and immunoprecipitation was performed with anti-Myc antibody. Phosphorylated EphrinB2 was detected by p-Tyr-specific antibody 4G10 and EphrinB2 was detected by anti-EphrinB2 antibody. All experiments were performed in triplicate and repeated 3 times.

Cell culture

Normal HUVECs and human umbilical arterial ECs (HUAEcs) were obtained from Cambrex (Walkersville, MD) and maintained in EGM2-supplemented medium (Invitrogen). For all experiments, HUVECs were used at passages 4 or below and collected from a confluent dish. SCC-15 and MCF-7 cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured under recommended conditions.

EC tube formation assay

Matrigel (250 μL; BD Biosciences, Palo Alto, CA) was placed in each well of an ice-cold 24-well plate. The plate was allowed to sit at room temperature for 15 minutes then incubated at 37°C for 30 minutes to permit Matrigel to solidify. HUVECs were prepared in EGM2 medium at a concentration of 1 × 10^5 cells/mL. The test protein was prepared at twice the desired concentration (5 concentration levels) in the same medium. Cells (500 μL) and protein (500 μL) were mixed and 200 μL of this suspension was placed in duplicate on the polymerized Matrigel. After 6-hour and 24-hour incubations, triplicate pictures were taken for each
concentration using a Bioquant Image Analysis system (Bioquant, Nashville, TN). Length of cords formed and number of junctions were compared among various groups. Experiments were performed in triplicate and repeated twice.

Cell adhesion assay
HUVECs were grown to 80% confluence and varying concentrations of sEphB4 were added to the supernatant overnight. Cells were prepared in EGM2MV medium at a density of $2 \times 10^4$ cells/mL and 250 $\mu$L cells were added to Matrigel-coated 96-well plates in the presence or absence of 10 nM PDGF-B. Fifteen minutes later, the supernatant was removed and wells washed with PBS. Fresh medium was added with 5 $\mu$L/mL MTT and absorbance measured in 2 hours as in cell viability assay.

Cell migration assay
Chemoattraction of HUVECs to VEGF- or bFGF-containing solution was assessed using a modified Boyden chamber, transwell membrane filter inserts in 24-well plates, 6.5 mm in diameter, 8 $\mu$m pore size, 10 $\mu$m thick, Matrigel-coated polycarbonate membranes (BD Biosciences). Cell suspensions of HUVECs ($2 \times 10^5$ cells/mL) in 200 $\mu$L medium were seeded in the upper chamber and 70 nM sEphB4 added simultaneously with stimulant (10-20 ng/mL VEGF or bFGF) to the lower compartment of the chamber. After incubation for 4 hours at 37°C, the upper surface of the filter was scraped with swab and filters were fixed and stained with Diff Quick. Ten random fields at $\times 40$ magnification were counted and the results expressed as mean number of cells. Negative nonstimulated control values were subtracted from stimulated control and protein-treated sample values, and the data were plotted as mean migrated cell plus or minus SD.

Cell viability assay
HUVECs, HUAECs, or cancer cell lines were plated in a 48-well plate at a density of $1 \times 10^4$ cells/well in a total volume of 500 $\mu$L. Medium was changed after cells attached and triplicate samples were treated with sEphB4 or CF2 (100 $\mu$L/mL) as described in “Results.” Cell viability was assessed by MTT as described previously.

Murine Matrigel plug angiogenesis assay
In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into a Matrigel plug containing the test sample. Matrigel rapidly forms a solid gel at body temperature, trapping the factors involved in angiogenesis. Matrigel (8.13 mg/mL, 0.5 mL) was mixed with single pellets was implanted and advanced toward the temporal corneal margin (within 0.7 ± 0.1 mm for bFGF pellets and 0.5 ± 0.1 mm for VEGF pellets). Erythromycin ointment was then applied to the operated eye to prevent infection and decrease surface irregularities. The subsequent vascular response was measured extending from the limbus vasculature toward the pellet. Data and clinical photos presented here were obtained on day 4 after pellet implantation.

Murine tumor xenografts
Tumor cells ($5 \times 10^6$ SCC-15 (head and neck squamous cell carcinoma cell line) or MCF-7 (human breast cancer cell line) were implanted subcutaneously in bilateral flanks of male athymic Balb/c nu/nu mice (6-8 weeks old), 5 mice/group. For assessing local effects of sEphB4, tumor cells were mixed with Matrigel (1:1 vol/vol; BD Biosciences) with or without 70 nM sEphB4. For assessing systemic effects, sEphB4 CF2 or PBS alone were injected intravenously daily, beginning on day 3 at a dose of 4 mg/kg. Tumor volume was measured 3 times a week estimated as $0.52 \times a \times b^2$, where $a$ and $b$ are the largest and smallest lengths of the palpable tumor. The Student $t$ test was used to compare tumor volumes, with $P < .05$ being considered significant. This experiment was done in triplicate with similar results in each experiment. Animals were humanely killed and tumors and normal organs harvested after 3 weeks. Harvested tissue was fixed in formalin for paraffin embedding and histologic analysis. All procedures were approved by our Institutional Animal Care and Use Committee and performed in accordance with the Animal Welfare Act regulations.
Results

Monomeric sEphB4 antagonizes forward and reverse signaling

The extracellular domain of EphB4 contains an N-terminal ligand-binding globular domain followed by a cysteine-rich domain and 2-fibronectin type III repeats. To identify the subdomain organization of the ectopic part of EphB4 necessary for the antiangiogenic activity, 2 deletion variants of EphB4 were produced and tested (Figure 1A): soluble EphB4 (sEphB4) protein, containing all 4 extracellular domains of the receptor, and its truncated variant lacking the ligand-binding domain (CF2). Both C-terminally His-tagged proteins were expressed in transiently transfected cultured mammalian cells and affinity purified to homogeneity from the conditioned media on Ni²⁺-chelate resin. The proteins migrate on SDS-PAGE higher than suggested by their predicted molecular weights of 57.8 kDa (sEphB4) and 41.5 kDa (CF2), due to glycosylation (Figure 1B).

To confirm that the purified sEphB4 recombinant protein was fully functional, equal amounts of sEphB4 and CF2 (10 pmol), as well as commercially available murine EphB4-Fc chimeric protein, were immobilized on beads. Equal aliquots of these beads (1% or 0.1 pmol) were used in an EphrinB2-AP–binding assay (Figure 1C), whereas the remaining beads were analyzed on SDS-PAGE (Figure 1B). As expected, sEphB4 and murine EphB4-Fc, but not CF2, bind EphrinB2-AP fusion protein as measured by AP enzymatic activity.

Recombinant sEphB4 and CF2 proteins were also tested for their ability to block EphrinB2-induced EphB4 phosphorylation in HUVECs (Figure 1D). Stimulation of HUVECs with EphrinB2-Fc fusion protein leads to a rapid induction of tyrosine phosphorylation of the EphB4 receptor. However, preincubation of the ligand with sEphB4, and to a lesser extent CF2, suppresses subsequent EphB4 activation, whereas sEphB4 and CF2 alone do not affect EphB4 phosphorylation status (Figure 1D left).

sEphB4 was also tested for its ability to block EphB4-induced phosphorylation of EphrinB2. 293T cells were transiently transfected with EphB4 or EphrinB2. Expression of transfected protein along with the lack of expression of both proteins in the parent cell line was confirmed by Western blotting (data not shown). EphB4- and EphrinB2-expressing 293T cell lines when cocultured induced phosphorylation of EphrinB2, which was blocked by the addition of sEphB4 (Figure 1E).

Effects of sEphB4 on HUVECs in vitro

Studies were performed to determine whether sEphB4 affects EC attachment, migration/invasion, proliferation, and tubule formation. HUVECs were plated on standard Matrigel-coated wells and treated with either sEphB4 or CF2 in the presence or absence of growth factors (see “Materials and methods”). sEphB4 inhibited tube formation (Figure 2A) in a dose-dependent manner (Figure 2B), whereas CF2 had no effect (Figure 2A). sEphB4, even at the lowest dose tested (2 nM), showed decrease in both the length of tubes and the number of junctions, with maximum effect seen at the highest concentration of 70 nM (Figure 2C). sEphB4...
maintained its inhibitory activity regardless of the growth factors used in the assay.

We then performed an in vitro invasion assay to measure the ability of HUVECs to degrade basement membrane and migrate toward a growth factor stimulus. sEphB4, but not CF2, significantly inhibited both bFGF and VEGF-induced migration by nearly 50% compared to control (Figure 2D). We then wished to assess if sEphB4 had an effect on EC attachment to basement membrane. To this end, HUAECs grown to 80% confluence were incubated with varying doses of sEphB4 overnight. Equal numbers of cells were allowed to plate on Matrigel-coated wells in the presence of 10 ng/mL PDGF-BB. PDGF-BB increased EC attachment 2-fold at 15 minutes. sEphB4 effectively inhibited this response in a dose-dependent fashion (Figure 2E). At a dose of 300 ng/mL, sEphB4 inhibited EC attachment by over 80% compared to control nonstimulated cells. A similar effect was observed at 5 minutes as well. Lastly, sEphB4 was found to have no detectable cytotoxic effect on HUVECs as assessed by MTT assay (data not shown).

**sEphB4 inhibits vascularization of Matrigel plugs in vivo**

To further demonstrate that sEphB4 can directly inhibit angiogenesis in vivo, we performed a murine Matrigel plug experiment. Matrigel supplemented with bFGF, VEGF, or both, with and without sEphB4, was injected into the ventral abdominal subcutaneous tissue of Balb/C nu/nu mice. Plugs without growth factors had virtually no vascularization after 6 days (Figure 3A top left). In contrast, plugs supplemented with bFGF or VEGF or growth factor along with CF2 (70 nM) had extensive vascularization and vessels with open lumen containing red cells throughout the plug (Figure 3A top and bottom right). Plugs containing 70 nM sEphB4 along with the growth factors had markedly reduced vascularization (Figure 3A bottom left) comparable to plugs without growth factor (Figure 3B). Furthermore, histologic examination showed decreased vessel staining in sEphB4-treated plugs. Fluorescence immunohistochemistry for CD31 confirms the endothelial origin of these cells, whereas the presence of EphB4+ and EphrinB2+ cells indicates that, as expected, both arterial and venous ECs migrate into the Matrigel in response to the growth factors (Figure 3C left panels). sEphB4 treatment reduced the numbers of CD31+, EphB4+, and EphrinB2+ as well (Figure 3C right panels). We quantitated the area of vascularization in 7 random blinded high-power fields using Spot v2.2.2 digital imaging system. sEphB4 treatment inhibited CD31+ vessels by 78%, EphB4+ vessels by 84%, and EphrinB2+ vessels by 64%.
sEphB4 inhibited corneal neovascularization

To further investigate the antiangiogenic activity of sEphB4 in vivo, we studied the effect of sEphB4 on bFGF- or VEGF-induced neovascularization in the mouse cornea. Growth factor–supplemented Hydron pellets implanted into a corneal micropocket induced angiogenesis (Figure 3D left). Neovascularization was markedly inhibited in mouse cornea with implanted pellets containing bFGF and sEphB4, in sharp contrast to the effect seen in corneas with pellets containing bFGF alone (Figure 3D right and left, respectively). Both the length of tubes and the number of junctions were significantly decreased in response to sEphB4 (Figure 3E). Similarly, the VEGF-induced vascular response was also significantly inhibited by sEphB4 (data not shown).

sEphB4 inhibited the growth of human tumors in athymic mice

Several tumor cell lines express EphB4 and increasing evidence indicates that EphB4 signaling may provide survival signals to these cells. To determine the effect of sEphB4 on the survival of tumor cells in vitro, tumor cells were cultured in the presence of varying concentrations of sEphB4. sEphB4 had no effect on tumor cell viability in vitro in all cell lines tested, including EphB4-expressing MCF-7, SCC-15, B16, PC3, and EphrinB2-expressing SLK cell lines (Figure 4A and data not shown).

Given the ability of sEphB4 to profoundly affect angiogenesis in vivo, we speculated that sEphB4 can inhibit tumor growth in vivo. We therefore examined the activity of sEphB4 in vivo in tumor xenograft models. MCF-7 (human breast carcinoma cell line) and SCC-15 (human squamous cell carcinoma of the head and neck) cells were premixed with Matrigel-containing vehicle or sEphB4 and implanted subcutaneously. Tumors in the control groups grew steadily over the treatment period, whereas xenografts supplemented with sEphB4 exhibited a significantly reduced growth rate (Figure 4B). MCF-7 tumor xenografts had a 62% reduction in final tumor volume and an 80% reduction in final tumor weight, whereas SCC-15 xenografts had a 54% and 64% reduction, respectively. Similar results were obtained in 2 additional independent experiments. Histologic analysis of tumors harvested at the time the animals were humanely killed (Figure 4C) showed large areas of necrosis as assessed by hematoxylin and eosin in the sEphB4-treated tumors. Tumor cell proliferation measured by Ki-67 staining was reduced by 70% in sEphB4-treated SCC-15 xenografts, with a 2.5-fold increase in apoptosis. In addition, sEphB4-treated tumor implants were markedly less vascularized (66% reduction) as assessed by CD31 vessels in tumor sections. Similar results were obtained in MCF-7 tumor xenografts (data not shown). Ki-67 staining was reduced by 65%, with a 4-fold increase in apoptosis and a 75% reduction in tumor microvasculature.

We next studied the effect of systemic administration of sEphB4 on tumor growth. SCC-15 and MCF-7 tumor xenograft-bearing mice were treated with intravenous sEphB4 at a daily dose of 10 mg/kg starting 4 days after tumor implantation. Administration of sEphB4 reduced MCF-7 tumor volume by over 70% and tumor weight by 82%, whereas CF2 inhibited tumor growth by 25% (Figure 4D). sEphB4 administration was well tolerated with no significant effect on body weight or the general well being of the animals, as determined by the absence of lethargy, intermittent hunching, tremors, or disturbed breathing patterns. Examination of
vital organs (lung, liver, kidney, heart) obtained when the animals were humanely killed showed no changes on histologic examination in sEphB4-treated mice (data not shown). sEphB4 reduced the number of proliferating tumor cells by 80%, increased apoptosis 4-fold, and diminished microvascular density by 75%. Similarly, SCC-15 tumor volume was reduced with sEphB4 treatment by 50% and tumor weight by 65% (data not shown). Proliferating cell number fell by 70%, apoptosis increased 2.8-fold, and tumor microvasculature was decreased by 77% (data not shown).

Discussion

EphrinB2 is the only ligand for EphB4 receptor and both proteins are essential for the development of the cardiovascular system. EphrinB2 is expressed in angioblasts destined to mature into arterial ECs and perivascular smooth muscle cells, whereas EphB4 defines venous lineage at the same step. However, neither protein is essential for this commitment because targeted disruption of either protein allows vasculogenesis to occur. Loss of either protein leads to arrest of angiogenesis leading to early embryonic lethality. Membrane localization of both proteins and, thus, signaling via cell-cell contact, with resultant dimerization and clustering of proteins on respective cells is required for optimal signaling. Dimeric or multimeric forms of the extracellular domain of EphrinB2 can replicate this effect leading to EphB4 receptor activation. Similarly, dimeric or multimeric forms of the extracellular domain of EphB4 can replicate this effect leading to EphrinB2 activation. We hypothesized that the soluble form of EphB4 (sEphB4) consisting of the extracellular domain, especially the N-terminus globular ligand-binding domain, would have an opposite function such that it would not only fail to induce phosphorylation of EphrinB2, but would also function as dominant negative, thus blocking forward and reverse signaling. sEphB4 indeed does not phosphorylate EphrinB2 and blocks ligand-induced EphB4 phosphorylation and receptor-induced ligand activation. Pursuant to the biochemical results, biologic studies fulfill the promise that sEphB4 blocks organization and migration of ECs to form tubules, which are the principal events leading to maturation of newly forming vessels. Importantly, sEphB4 exerts similar effects in angiogenesis models in vivo, and further, significantly inhibits tumor growth as well. Another recombinant protein, CF2, which lacks the globular ligand-binding domain, was also tested. Interestingly, this protein partially inhibits EphB4 phosphorylation by EphrinB2 and also has a small inhibitory effect on tumor growth in vivo. Given that this protein does not inhibit ligand binding, the
Angiogenesis involves the recruitment and attachment of ECs at sites of vascularization and their proliferation and organization into tubelike structures. We show here that sEphB4 interrupts 2 of these 3 processes—prevents EC attachment to extracellular matrix proteins and their organization to form tubelike structures. EphB4, like other Eph receptors, may also interact with other cell membrane proteins as exemplified by the interaction of EphB2 with glutamate receptors and possibly other yet unidentified interactions. It is conceivable that sEphB4 competes not only with EphrinB2/EphB4 interactions but also other extracellular protein interactions. Along these lines, we show that sEphB4 can inhibit PDGF-induced EC attachment. Eph receptor signaling has been shown to modulate focal adhesion kinases and modulates several members of the integrin family of proteins. These proteins are also targets of PDGF-R signaling. Thus, interruption of EphB4 signaling by sEphB4 may modulate integrin-matrix interaction, similar to that seen with disruption of EphB1 activation and thus reduce cell attachment and tube formation.

Loss of EphB4 in the embryo permits the primary vascular complex to develop. However, the vessels fail to mature, such that all vessels have small and similar luminal diameter, and lack communication between arterial and venous capillary networks. We demonstrate similar effects with sEphB4 in vivo. Growth factors such as bFGF or VEGF or a combination of factors when impregnated in Matrigel recruit ECs and produce large, branching vessels integrated into the circulation with red blood cells in the lumen and degradation of surrounding matrix. Rudimentary vessel-like structures seen in sEphB4-containing Matrigel plugs indicates inhibition of effective EC migration resulting in few, short vessel-like structures with narrow or no lumen, lacking red cells. EphB4 antagonizes equally well the angiogenic response to several different vascular growth factors including VEGF and bFGF. This is consistent with the role of EphB4/EphrinB2 in embryonic vessel maturation, which is common to several proangiogenic stimuli. sEphB4 is also highly effective in laser injury-induced models of choroidal neovascularization in which numerous factors are induced simultaneously.

Tumor growth and metastasis are dependent on angiogenesis via release of various proangiogenic factors. Maturation of tumor vessels is dependent on EphB4-EphrinB2 interactions and thus subject to modulation. Tumor growth of 2 distinct epithelial tumor types was markedly reduced in response to sEphB4 with a profound inhibition in tumor microvasculature. Thus, sEphB4 significantly influences tumor-induced vascular response and secondarily, tumor growth. Tumor xenografts also showed reduced proliferation and increased apoptosis following sEphB4 treatment. EphB4 and EphrinB2 are not known to influence cell proliferation or cell survival and sEphB4 does not have an impact on tumor cell survival in vitro. Hence, these effects on tumor growth are likely secondary to reduced blood supply. The possibility that EphB4, EphrinB2, or other EphrinB2-activating Eph receptors, when expressed in tumor cells, provide survival signals and thus subject to modulation needs further investigation. Similarly, it is well known that stroma plays a critical role in the growth and progression of tumors. It is also likely that sEphB4 modulates the interaction between tumor cells and stroma or tumor cells and matrix proteins released from the stroma. Lastly, tumor growth is modulated by the innate immune system even in athymic mice. The role played by tumor cell-expressed EphB4 or EphrinB2 on innate immune responses has not yet been investigated.

A recent report by Martiny-Baron et al showed that interruption of EphB4-EphrinB2 signaling can inhibit angiogenesis and tumor growth. Our studies add further mechanistic insights into these effects and further establish sEphB4 as a candidate for therapy in vascular proliferative disorders and cancers. First, we provide documentation that sEphB4 interrupts ligand-induced receptor phosphorylation and receptor-induced ligand phosphorylation. Further, sEphB4 inhibits EC attachment and tube formation in vitro in the absence of exogenous EphB4, regardless of the growth factor that is driving angiogenesis, data that better reflect the known effects of EphB4 knockdown in the embryo. We extend these findings to show that sEphB4 can similarly inhibit angiogenesis in vivo in different models of adult neovascularization. Lastly, our tumor studies in different tumor types using systemic administration of sEphB4 demonstrate that sEphB4 can reach tumor tissue and hence has potential for clinical application.

In summary, the monomeric form of sEphB4 ectodomain displays inhibitory effects on the following: (1) forward and reverse signaling by EphB4 and EphrinB2; (2) EC adhesion, migration, and tube formation; (3) growth factor- or tumor-derived angiogenesis; and (4) tumor growth accompanied by reduced proliferation and increased apoptosis. sEphB4 is thus a candidate for therapeutic assessment in angioproliferative disorders such as macular degeneration and cancer.

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References
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