Heparin potentiates Avastin-mediated inhibition of VEGF binding to fibronectin and rescues Avastin activity at acidic pH

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Vascular endothelial growth factor-A (VEGF) plays a critical role in stimulating angiogenesis in normal and disease states. Anti-VEGF antibodies have been developed to manage pathological angiogenesis. Bevacizumab, sold under the brand name Avastin, is a humanized mAb that binds VEGF and blocks its binding to its signaling receptor, VEGFR-2, and is used to treat patients with a variety of cancers or retinal disorders. The ability of Avastin to modulate other nonreceptor interactions of VEGF has not been fully defined. In this study, we investigated Avastin’s capacity to modulate VEGF165 binding to porcine aortic endothelial cells and to heparin and fibronectin (FN) across a range of pH values (pH 5–8). We observed that Avastin slightly enhanced VEGF binding to heparin and that heparin increased VEGF binding to Avastin. In contrast, Avastin inhibited VEGF binding to cells and FN, yet Avastin could still bind to VEGF that was bound to FN, indicating that these binding events are not mutually exclusive. Avastin binding to VEGF was dramatically reduced at acidic pH values (pH 5.0–6.5), whereas VEGF binding to FN and nonreceptor sites on cells was enhanced. Interestingly, the reduced Avastin–VEGF binding at acidic pH was rescued by heparin, as was Avastin’s ability to inhibit VEGF binding to cells. These results suggest that heparin might be used to expand the clinical utility of Avastin. Our findings highlight the importance of defining the range of VEGF interactions to fully predict antibody activity within a complex biological setting.

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The abbreviations used are: VEGF, vascular endothelial growth factor; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; PBS, fetal bovine serum; FGFR-1, fibroblast growth factor receptor 1; FN, fibronectin; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PAEC–EV, porcine aortic endothelial cell–empty vector; PAEC–FLK, porcine aortic endothelial cell with VEGFR-2 expression; PBS-B-T, PBS with bovine serum albumin and Tween 20; 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide; VEGFR-2, VEGF receptor 2.

Major mediator of angiogenesis through its ability to bind to and activate receptors, specifically VEGF receptor 2 (VEGFR-2), on the surface of endothelial cells to signal cell migration, proliferation, differentiation, and survival (2, 3). VEGF–VEGFR-2 interactions are further enhanced by the presence of heparin/heparan sulfate (HS) proteoglycans (4). Heparin is produced in mast cells, whereas HS is found on cell surfaces and in the extracellular matrix (ECM) linked to proteins such as HSPGs. Heparin/heparan sulfates are involved in many biological processes through the ability to interact with numerous proteins such as growth factors, cytokines, cell-surface proteins, enzymes, and ECM proteins (5–7). Within the VEGF system, heparin/HS participates in modulating binding interactions at many levels (4). Heparin/HS directly binds to VEGF, enhances the affinity of VEGF binding to VEGFR-2, and binds to the following VEGF-binding proteins: VEGF receptor 1, neuropilin 1, and fibronectin (FN). Thus, heparin/HS has the potential to modulate VEGF activity through a variety of mechanisms and will likely prove to influence the effectiveness of angiogenic and anti-angiogenic therapies aimed at modulating the VEGF growth factor system.

In 1997, a humanized version of an anti-VEGF mAb, bevacizumab, commercially known as Avastin, was developed to bind VEGF and block its binding to VEGFR-2 (8–10). Avastin was shown to be effective at reducing angiogenesis in many clinical settings leading to FDA approval for the treatment of cancers such as ovarian cancer, advanced cervical cancer, metastatic renal cell carcinoma, recurrent glioblastoma, advanced nonsquamous nonsmall cell lung cancer, and metastatic colorectal cancer (9). However, Avastin does not prevent VEGF binding to heparin or neuropilin 1, another cell-surface VEGF-binding protein (11). Neuropilin enhances VEGF binding to its signaling receptor, VEGFR-2 (4, 12). Avastin forms multimeric complexes with VEGF as well as binds Fcγ receptors in the presence of heparin (13, 14). This in turn activates platelet aggregation as well as a cell death cascade (15). The ability of VEGF to bind heparin and neuropilin in the presence of Avastin is believed to limit VEGF clearance by Avastin. HSPGs and neuropilin on endothelial cell surfaces and within the ECM are believed to trap VEGF, thus limiting the effectiveness of Avastin (16).

In addition to HSPGs, VEGF also binds to the ECM protein FN within the hep 2 domain (type III domains 12–14), and this interaction enhances angiogenic activity of VEGF (17–20). Growth factor interactions with ECM are associated with localized targeted signaling (21, 22). In addition, FN interaction with...
VEGF is able to facilitate a synergistic binding complex with VEGFR-2 and the $\alpha v$3 integrin that potentiates the angiogenic activity (23–26). Heparin/HS also binds transiently to the hep 2 domain of FN causing a rearrangement of FN leading to exposure of the VEGF-binding site. The ability of heparin/HS to catalyze the conversion of FN from a closed to an open conformation allows VEGF and other growth factors to decorate the ECM and to locally guide cell response (20, 27). Interestingly, the binding of VEGF to FN is enhanced at acidic pH (18, 28) suggesting that this process would be more pronounced in hypoxic and locally acidic extracellular tissue environments. Although the ability of Avastin to modulate a variety of VEGF-binding events has been explored, little is known about how VEGF–FN interactions and changes in extracellular pH might influence the activity of Avastin.

In this paper, we investigated the ability of Avastin to influence VEGF binding to FN and to the nonreceptor sites of endothelial cells, and we explored the ability of heparin to modulate this process. Using an array of cell-free and cell-based binding assays, we found that Avastin partially inhibited VEGF binding to purified FN and to cells and that this activity was dramatically attenuated at acidic pH. Moreover, the pH-dependent loss of Avastin inhibitory activity was correlated with a reduction in VEGF–Avastin binding at acidic pH. These results suggest that the addition of heparin might expand the clinical utility of Avastin. Taken together, these findings highlight the importance of defining the range of interactions of an antibody target under a variety of conditions to fully predict antibody activity within a complex biological setting.

**Results**

**Avastin inhibits VEGF binding to nonreceptor-binding sites on endothelial cells**

Avastin is a mAb against VEGF that is able to prevent VEGF binding to VEGFR-2 (8). This effect is illustrated in Fig. 1A where Avastin inhibited $^{125}$I-VEGF binding to immobilized Fc-VEGFR-2 by >95% at the highest dose tested. Although this mechanism of action leads to inhibition of VEGFR-2 signaling, the complexity of VEGF interactions with other components on and around endothelial cells likely contributes to the inconsistent effectiveness of Avastin. To examine the ability of Avastin to modulate VEGF binding on endothelial cells, we utilized cells engineered to express VEGFR-2 (PAEC–FLK) (Fig. 1B) and noted that the same concentration range of Avastin inhibited $^{125}$I-VEGF binding to ~15% of that in the absence of Avastin at the highest concentration tested. The fact that Avastin did not inhibit binding to cells to the same extent as with purified VEGFR-2 is potentially a reflection of the fact that VEGF binds to nonreceptor components, in addition to VEGFR-2, on these cells, namely to HS and FN within the extracellular matrix and...
on the cell surface that might not be affected by Avastin to the same extent as VEGFR-2.

To explore the ability of Avastin to influence VEGF binding to nonreceptor components, we conducted selective extraction of the cells using a high-salt solution that releases VEGF from HS and FN but not VEGF receptors. Furthermore, analysis was done with VEGFR-2–negative and -positive cells (PAEC–EV and PAEC–FLK, respectively) to ensure that the binding being measured was VEGFR-2–independent. The presence of Avastin inhibited the binding of 125I-VEGF to nonreceptor sites on PAEC–EV and PAEC–FLK cells by 40% suggesting that some nonreceptor components may be resistant to Avastin inhibition (Fig. 1C). We also included a condition where cells were pretreated with heparin to expose VEGF-binding sites on FN (28). The heparin pretreatment and washing protocol have previously been demonstrated to remove all detectable heparin; thus, the increased binding observed after pretreatment is directly reflective of increased VEGF binding to FN (28, 29). As such, salt-extractable binding of VEGF in cells subjected to heparin pretreatment will be enriched for VEGF bound to FN. Heparin-pretreated cells showed increased (2-fold) binding compared with their respective nonpretreated controls. Interestingly, Avastin showed greater relative inhibition of VEGF binding to the nonreceptor sites on cells that were pretreated with heparin suggesting that its ability to reduce this binding mainly lies in its ability to inhibit VEGF interactions with FN. Subsequently, including heparin in the binding buffer to compete for VEGF binding to FN caused a reduction in VEGF binding to the ECM that was more pronounced in the heparin-pretreated cells. The combination of Avastin and heparin caused a further decrease in VEGF binding to the nonreceptor sites on these cells. These results suggest that Avastin may selectively interfere with VEGF binding to FN as opposed to HS sites on endothelial cells.

Avastin does not inhibit VEGF binding to heparin

To evaluate the action of Avastin on the nonreceptor-binding sites for VEGF, we employed cell-free assays. To represent HS and FN but not VEGF receptors. Furthermore, analysis was done with VEGFR-2–negative and -positive cells (PAEC–EV and PAEC–FLK, respectively) to ensure that the binding being measured was VEGFR-2–independent. The presence of Avastin inhibited the binding of 125I-VEGF to nonreceptor sites on PAEC–EV and PAEC–FLK cells by 40% suggesting that some nonreceptor components may be resistant to Avastin inhibition (Fig. 1C). We also included a condition where cells were pretreated with heparin to expose VEGF-binding sites on FN (28). The heparin pretreatment and washing protocol have previously been demonstrated to remove all detectable heparin; thus, the increased binding observed after pretreatment is directly reflective of increased VEGF binding to FN (28, 29). As such, salt-extractable binding of VEGF in cells subjected to heparin pretreatment will be enriched for VEGF bound to FN. Heparin-pretreated cells showed increased (2-fold) binding compared with their respective nonpretreated controls. Interestingly, Avastin showed greater relative inhibition of VEGF binding to the nonreceptor sites on cells that were pretreated with heparin suggesting that its ability to reduce this binding mainly lies in its ability to inhibit VEGF interactions with FN. Subsequently, including heparin in the binding buffer to compete for VEGF binding to FN caused a reduction in VEGF binding to the ECM that was more pronounced in the heparin-pretreated cells. The combination of Avastin and heparin caused a further decrease in VEGF binding to the nonreceptor sites on these cells. These results suggest that Avastin may selectively interfere with VEGF binding to FN as opposed to HS sites on endothelial cells.

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by simultaneously binding to heparin and VEGF. To gain insight into how Avastin influences VEGF binding to heparin, Avastin was incubated with heparin-coated surfaces with and without VEGF, and the amount of Avastin associated with the plate was measured with a horseradish peroxidase–linked secondary antibody using a standard ELISA protocol. VEGF alone was included as a negative control to determine the background level of secondary antibody binding to VEGF–heparin complexes. There was no detectable binding of Avastin to the heparin plate in the absence of VEGF (Fig. 2B). However, the addition of VEGF resulted in a significant signal indicating that VEGF–Avastin complexes are able to bind to heparin.

We further evaluated the influence of heparin on VEGF binding to Avastin by immobilizing Avastin to protein A–coated plates and allowing soluble 125I-VEGF to interact with the Avastin surface. A chimeric form of fibroblast growth factor receptor, Fc-FGFR1, was also bound to the protein A plate as a control for potential nonspecific interactions of VEGF with the Fc component of Avastin. 125I-VEGF binding to immobilized Avastin was significantly enhanced in the presence of heparin, with a maximal effect (>2-fold) being observed in the presence of 0.1 μg/ml heparin (Fig. 2C). There was no detectable binding of 125I-VEGF to the immobilized Fc-FGFR1 chimera. Thus, it appears that the binding of heparin to VEGF enhances VEGF binding to Avastin, whereas VEGF binding to heparin is enhanced by Avastin. Taken together, these results indicate that heparin, VEGF, and Avastin are able to form a high-affinity ternary complex, whereby the individual molecular interactions (VEGF with heparin and VEGF with Avastin) positively influence one another.

Avastin inhibits VEGF binding to FN and binds to FN in the presence of VEGF

Although Avastin was unable to inhibit VEGF binding to heparin, Avastin at 1–100 nM inhibited VEGF binding to FN by 35–45% (Fig. 3A). Although this level of inhibition was statistically significant, it was modest compared with that observed with VEGF-2, indicating that VEGF binding to Avastin and FN might not be mutually exclusive. Thus, Avastin binding to FN was evaluated in the presence and absence of VEGF (Fig. 3B). Although there was detectable Avastin associated with the FN surface in the absence of VEGF at the highest Avastin concentrations tested, the levels were dramatically enhanced by VEGF, suggesting that Avastin is able to bind to VEGF while it is complexed to FN. Taken together, these results suggest that VEGF–Avastin complexes are able to bind to FN, but with reduced affinity when compared with VEGF alone.

Inhibition of VEGF binding to FN by Avastin and binding of VEGF to Avastin are decreased at acidic pH

VEGF binding to FN is enhanced at acidic pH values; thus, we investigated the ability of Avastin to modulate VEGF binding to FN at a range of pH values (pH 5–8). Consistent with previous studies, VEGF binding to FN increased as the pH decreased; but interestingly, the relative ability of Avastin to inhibit VEGF–FN binding was reduced at acidic pH (Fig. 4, A and B). Whereas Avastin inhibited VEGF binding to FN by ~80% at pH 8, it only inhibited binding by ~10% at pH 5. The reduced ability of Avastin to inhibit VEGF binding to FN under acidic conditions could reflect an alteration in VEGF–Avastin interactions under low pH conditions. Thus, direct binding of VEGF to immobilized Avastin was measured across this range of pH values, and we observed an ~10-fold reduction in VEGF binding to Avastin as the pH dropped from 8 to 5 (Fig. 4C).

Heparin rescues Avastin–VEGF binding at low pH and enhances the inhibition of VEGF binding to cells

Soluble heparin inhibits VEGF binding to FN (18, 29), and we observed increased inhibition of binding to PAECs with the combination of Avastin and heparin compared with each alone (Fig. 1C). We also found that heparin increased VEGF binding to Avastin (Fig. 2C). Thus, we tested whether heparin is able to enhance Avastin–VEGF binding at low pH values. Binding of 125I-VEGF to immobilized Avastin was measured in the presence and absence of heparin at pH 7.5 and 5.5 (Fig. 5). As observed above, the binding of 125I-VEGF to Avastin was significantly reduced at pH 5.5 compared with that at pH 7.5. However, there was no difference between VEGF–Avastin binding under the two pH conditions when heparin was present suggesting that heparin is able to rescue VEGF–Avastin binding under acidic conditions.
125I-VEGF binding to PAEC–FLK cells was measured to determine whether the ability of heparin to overcome the effects of acidic conditions on Avastin–VEGF binding translates to enhanced inhibition of nonreceptor and VEGFR-2 binding within a complex cellular context. Measurements were conducted at pH 5.5, pH 6.5, and pH 7.5 as it was across this range of pH values where we observed maximal alterations in Avastin activity (Figs. 4 and 5).

125I-VEGF binding to the nonreceptor and receptor components on PAEC–FLK were sequentially measured using selective extraction methods. After 125I-VEGF was allowed to bind to the endothelial cell monolayers, a high-salt solution was used to extract the fraction of 125I-VEGF bound to nonreceptor components, as described in Fig. 1. The remaining bound 125I-VEGF, which includes the fraction bound to VEGFR-2 on PAEC–FLK cells, was extracted by solubilizing the cell layer in NaOH (Fig. 6). VEGF binding to nonreceptor sites was enhanced at pH 5.5 compared with pH 6.5 and 7.5 (18.3 fmol at pH 5.5; 8.1 fmol at pH 6.5; and 1.4 fmol at pH 7.5 bound per well, respectively), whereas the ability of Avastin to inhibit VEGF binding was reduced (36.9% inhibition at pH 5.5; 74.3% inhibition at pH 6.5; and 68.3% inhibition at pH 7.5) (Fig. 6A). However, the addition of heparin with Avastin dramatically enhanced inhibition under all pH conditions (91.5% inhibition at pH 5.5; 95.2% inhibition at pH 6.5; and 85.7% inhibition at pH 7.5), greater than with heparin alone.
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Figure 6. Heparin enhances Avastin inhibition of VEGF binding to endothelial cells at low pH. PAEC–FLK cells were pretreated with heparin (10 μg/ml) for 1 h at 4°C to expose VEGF-binding sites, washed, and then incubated with 125I-VEGF (10 ng/ml) ± heparin (1 μg/ml) ± Avastin (1 nM) at pH 5.5, 6.5, and 7.5 for 2 h at 4°C. The 125I-VEGF bound to nonreceptor sites (A) and salt-resistant sites (receptor) (B) were extracted and measured as described under “Experimental procedures.” The level of VEGF bound per well in each fraction is presented as the average of triplicate determinations ± S.D. All conditions in A were statistically significantly different from one another at each pH value and were different from each other at the three pH conditions. B, all conditions except heparin versus control at pH 7.5 were different from one another at each pH, and all except Avastin alone and Avastin and heparin between pH 5.5 and 6.5 were different from each other at the pH values tested.

(62.5% at pH 5.5; 58.3% at pH 6.5; and 25% at pH 7.5). Although Avastin was less active at low pH values and showed greater inhibition at high pH values, heparin showed the opposite trend. Overall, the data indicate that the presence of heparin compensates for the loss of Avastin–VEGF binding at low pH, thus providing a means to restore Avastin-mediated inhibition under these conditions.

Discussion

Anti-VEGF therapies have improved outcomes for a variety of indications, including cancer and retinal disease (30, 31). One of the most widely used anti-VEGF drugs is bevacizumab (Avastin), which was first given FDA approval in 2004 for combination use with chemotherapy. It has since been approved for a number of cancers and is also used off-label for the treatment of age-related macular degeneration and other retinal disorders (32, 33). Although Avastin is a potent inhibitor of VEGF binding to VEGFR-2, it binds to human retinal epithelial cells and human umbilical vein endothelial cells in the presence of VEGF (11, 15). These findings suggest that Avastin–VEGF complexes remain capable of binding to nonreceptor-binding sites on cells and within the ECM. Indeed, these same studies also noted that VEGF is still able to bind neuropilin and heparin in the presence of Avastin and predicted that VEGF clearance was low due to being trapped by these VEGF-binding molecules (11). Thus, in this study, we investigated the ability of Avastin to modulate VEGF binding to the ECM protein FN, and we observed that Avastin partially inhibited VEGF binding to FN (Fig. 3A) and that Avastin–VEGF complexes were able to bind to FN (Fig. 3B). Interestingly, the ability of Avastin to inhibit VEGF binding to cells was enhanced by the addition of heparin, especially at acidic pH where Avastin–VEGF interactions were dramatically attenuated (Figs. 5 and 6). Taken together, our findings suggest that binding of VEGF to the ECM, specifically FN, may trap VEGF in locally acidic regions of tissue where it will interfere with the ability of Avastin to effectively clear and inactivate VEGF.

We and others have previously demonstrated that pretreatment of cells and FN with heparin leads to increased VEGF binding as a result of the ability of heparin to stably modify FN structure (20, 28, 29). However, the addition of soluble heparin along with VEGF in the binding assay reduced VEGF binding to nonreceptor sites, in the presence or absence of heparin pretreatment, and showed an additive effect with Avastin (Fig. 1C). Interestingly, heparin enhanced VEGF binding to Avastin, and Avastin enhanced VEGF binding to heparin, suggesting that Avastin, VEGF, and heparin form a cooperative high-affinity ternary complex. These findings suggest the potential that a composite of heparin and Avastin might function as a potent VEGF inhibitory agent that can target both receptor and nonreceptor binding.

While it is known that VEGF binding to the FN matrix is higher at lower pH conditions (18), these same conditions are also connected to triggering proangiogenic signals (34-36). The growth of tumors beyond a 1-mm diameter leaves the tumor center deprived of nutrients, which not only leads to low O2 levels but also to low extracellular pH conditions (36). As these low pH conditions progress, the probability of VEGF binding to FN increases. This process can lead to enhanced VEGF deposition within the tumor microenvironment leading to a highly-directed biochemical gradient (21, 22). FN matrix–bound VEGF may then become available to bind and activate VEGFR-2 on endothelial cell surfaces. Indeed, we and others have previously demonstrated that VEGFR-2 and FN can simultaneously bind to VEGF suggesting a mechanism by which cells can respond to ECM-associated VEGF (37). Although anti-VEGF therapies such as Avastin are able to target VEGF to prevent its binding to VEGFR-2 (9), we found that Avastin can associate with FN in the presence of VEGF. Thus, unlike with VEGFR-2, Avastin binding to VEGF does not appear to block the FN-binding site on VEGF, but instead it appears to reduce the affinity of the VEGF–FN interaction. Additionally, low pH conditions hinder VEGF and Avastin binding (Fig. 4), which limits the ability of Avastin to inhibit VEGF binding to ECM and VEGFR-2 (Fig. 6). These findings identify an intrinsic limitation in the Avastin mechanism of action that may hinder activity under certain conditions.

The prospects of treating disease by targeting pathological angiogenesis have become a reality that has extended and improved the lives of patients (38). In particular, the use of
anti-VEGF antibodies and other binding proteins targeting VEGF demonstrates the value of targeting VEGF for anti-angiogenic therapy. Unfortunately, most anti-angiogenic drugs, such as Avastin, have shown inconsistent results and somewhat limited clinical success in certain settings. The findings herein showing that Avastin binding to VEGF was dramatically reduced at acidic pH while binding to the FN was enhanced suggest that this limitation might be compounded in hypoxic and locally acidic tissue regions. The observation that heparin rescued the pH-dependent loss of Avastin–VEGF binding and was able to enhance the inhibitory effects of Avastin on VEGF binding to receptor and nonreceptor sites suggests the intriguing possibility that heparin, or other potentially more specific polysaccharides, might be used in combination with Avastin to improve its clinical utility. The development of more effective and specific anti-VEGF therapies for the treatment of pathological angiogenesis will need to consider the influence of VEGF binding to the ECM and the context of the particular tissue being targeted for treatment.

**Experimental procedures**

**Reagents**

Streptavidin-coated 96-well plates and protein A–coated plates were from Pierce. Avastin (bevacizumab) was from Midwinter Solutions Ltd. (Staffordshire, UK). Heparin was from porcine mucosa; human plasma FN and biotin–heparin were from Millipore Sigma. Cell culture agents such as Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin (1:1) mixture, and L-glutamine were purchased from Corning (Tewkesbury, MA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Atlanta, GA). Recombinant human VEGF165 and Fc-FGFR1 chimeric protein were purchased from R&D Systems (Minneapolis, MN). 125I-Bolton-Hunter’s reagent was purchased from PerkinElmer Life Science and was used to label the VEGF as described previously (39). Radioactive 125I in samples was measured with a Cobra Auto-Gamma 5005-counter (Packard Instruments, Meridian, CT). Blocker casein in phosphate-buffered saline (PBS) and BCA assay kit were obtained from Thermo Fisher Scientific (Waltham, MA). Peroxidase-AffiniPure donkey anti-human IgG, Fcγ fragment-specific was obtained from Jackson ImmunoResearch (West Grove, PA). KPL TMB Microwell peroxidase substrate system was purchased from SeraCare (Milford, MA).

**VEGFR-2–binding assays**

A 96-well polystyrene plate was coated with 5 nM VEGFR-2 in PBS overnight at 4 °C on a shaker. Adsorbed VEGFR-2 wells were washed twice with 0.1% BSA, 0.05% Tween 20 in PBS (PBS-B-T). 125I-VEGF (10 ng/ml) with various concentrations of Avastin (0, 1, 10, and 100 nM) in PBS-B-T was added to the VEGFR-2–coated wells and incubated for 2 h at 4 °C with shaking. After the binding reaction, the unbound 125I-VEGF was removed by washing each well three times with PBS-B-T. Bound 125I-VEGF was then extracted from the plate surface by incubating each well with 100 µl of 1 N NaOH for 5 min followed by an additional 100 µl of 1 N NaOH. The radioactivity in each sample was counted.

**Heparin-binding assays**

Streptavidin-coated 96-well plates were incubated with 5 µg/ml biotin–heparin or 1 µg/ml biotin in PBS overnight at 4 °C on a shaker (100 µl/well). Unbound biotin or biotin–heparin was washed away the next day, and combinations of 125I-VEGF with and without Avastin were added to each well in 100 µl of 0.1% BSA, 0.05% Tween 20 in PBS (PBS-B-T). The treatments were incubated for 2 h at 4 °C on a shaker, and then the unbound material was removed by washing each well three times with binding buffer. Bound 125I-VEGF was then extracted from the plate surface with 100 µl of 1 N NaOH for 5 min followed by an additional 100 µl of 1 N NaOH. The radioactivity in each sample was counted.

**Avastin-binding assays**

Protein A–coated plates were incubated with either Avastin or Fc-FGFR-1 (0.5 nM; 100 µl/well) in PBS-B-T for 2 h at room temperature on a shaker. The unbound protein was removed by washing each well three times with PBS-B-T, and then 125I-VEGF ± heparin was added to each well and incubated for 2 h on a shaker at room temperature. Control experiments were conducted where heparin (100 µg/ml) was added to PBS-B-T and the pH was measured, and no difference was observed compared with PBS-B-T alone. 125I-VEGF bound to the surface of each well was then extracted two times with 100 µl of 1 N NaOH for 5 min each. The radioactivity in each sample was counted.

**Fibronectin-binding assays**

96-Well polystyrene plates were coated with a mixture of 10 µg/ml FN and 10 µg/ml heparin in PBS overnight at 4 °C on a shaker. The heparin was removed from the FN adsorbed to the plate surface by washing each well three times with PBS, and the FN surfaces were incubated with combinations of 125I-VEGF ± Avastin in 125 mM NaCl, 25 mM HEPES, 0.01% BSA (100 µl/well) for 2 h at room temperature. VEGF bound to the FN matrix was either extracted with 1 N NaOH and counted (as described above), or an ELISA protocol was used to measure bound Avastin levels. For ELISA, the wells were blocked with 1.5% BSA, 0.2% casein in 1× PBS for 1 h at room temperature on the shaker. The wells were incubated with 1:10,000 donkey anti-human IgG in blocking buffer for 35 min, then incubated with TMB substrate for 10 min, and stopped with 1 N phosphoric acid. The absorbance was read at 450 and 570 nm on a Spectromax plate reader. A570 nm values were subtracted from A450 nm values to account for background. The samples were collected in tubes, and each well was then incubated twice with 100 µl of 1 N NaOH for 5 min each, and the bound 125I-VEGF was extracted and counted.

**VEGF binding to endothelial cells**

PAECs (passage 4–15) that have been engineered to express VEGFR-2 (FLK cells) or transformed with an empty vector (EV cells) were the gift from Nader Rahimi at the Boston University School of Medicine. PAECs were maintained in DMEM containing 10% FBS, 1% penicillin/streptomycin mixture, and 2 mM L-glutamine. For binding assays, the cells were plated at 30,000 cells/well in 24-well plates and incubated overnight. The fol-
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lowing day, the medium was changed to 0.1% FBS-containing media, and the cells were allowed to incubate for an additional 24 h. After starvation, the cells were washed three times with cell-binding buffer (DMEM, 25 mM HEPES, 0.1% BSA, pH 6.5) and incubated on ice for 10 min in the last wash to inhibit receptor internalization. Mixtures of $^{125}$I-VEGF, heparin, and Avastin in the cell-binding buffer were added to the cells and incubated for 2 h at 4 °C. Control experiments demonstrated that the addition of heparin did not alter the pH of the binding buffer. Control experiments were done to ensure that the addition of heparin to the binding buffer did not alter the pH. The cells were washed three times with cell-binding buffer to remove unbound $^{125}$I-VEGF, and the ECM was extracted with a high-salt buffer (1 M NaCl, 25 mM HEPES) followed by PBS (29). The remaining cell-bound $^{125}$I-VEGF was extracted with 200 μl of 1 N NaOH twice for 5 min each and counted. This was accompanied by an MTT assay to quantify the cell density of both cell lines on a separate plate to normalize binding to the relative cell number.

MTT proliferation assay

To evaluate the cell density 2 days after plating, cell number was determined using an MTT proliferation assay kit (ATCC, Manassas, VA). The protocol followed the manufacturer’s instructions where the cells were washed and then incubated with the MTT reagent for 2 h at 37 °C on the shaker. This was followed by addition of detergent to each well.

The cell extract was incubated for another 2 h at room temperature on the shaker. Absorbance was measured at 570 nm on the Spectromax plate reader. The $^{125}$I-VEGF–bound levels were normalized based on the cell MTT absorbance values.

Statistical analysis

The data presented in the figures represent the mean of triplicates ± S.D. values. All experiments were repeated at least three times. To evaluate the statistical significance of the results, data were subjected to single-factor ANOVA. The differences were considered to be significant when the p values were found to be <0.05.

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