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Regulating specific growth factor signaling using immobilized branched ligands

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Direct targeting of growth factor signaling pathways has become an important means of treating a variety of medical conditions. Antibodies and other molecules^[1] that directly bind to growth factors and receptors have been developed to treat diseases that are characterized by elevated levels of a particular growth factor, such as macular degeneration.^[2] While effective in downregulating specific signaling pathways, these treatments can readily diffuse away from the desired treatment site, which is problematic for two reasons. First, the effectiveness of a given dose diminishes over time as the therapeutic molecules diffuse away from the site of interest. Second, the molecules that diffuse away from the site of interest are often still biologically active and can cause undesirable side effects elsewhere in the body.

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Multiple investigators are working to develop biomaterials that can provide spatial localization, in addition to the therapeutic action that current antibody-based treatments provide.^[3] For example, peptide ligands identified via phage display have recently been immobilized within hydrogels and used in one study to locally release nerve growth factor,^[4] and in another study to locally sequester tumor necrosis factor- α .^[5] Heparin and other naturally occurring molecules that bind VEGF have also been incorporated into biomaterials in an attempt to regulate growth factors^[6–7]. These represent promising approaches for specific growth factor regulation, but there remains a need to develop new approaches to achieve highly efficient growth factor regulation, while also maintaining a high level of specificity. Here we hypothesized that branched ligands derived from a growth factor receptor could be used to achieve highly efficient and specific growth factor sequestering, in view of the prevalence of multivalency in natural growth factor-receptor binding.

A variety of growth factor-receptor interactions are dependent on dimerization, as many growth factors exist as disulfide-linked dimers, and growth factor receptor activation typically involves receptor dimerization. For example, the most common isoforms of VEGF, including VEGF₁₆₅, exist as disulfide-linked dimers, and VEGF receptor 2 (VEGFR2) dimerizes upon VEGF binding. We hypothesized that a VEGF binding ligand derived from VEGFR2 would show enhanced VEGF binding affinity when presented as a dimer - in a manner that mimics the dimerization of VEGFR2 during natural VEGF signaling. To test this hypothesis we synthesized a series of poly(ethylene glycol) (PEG) hydrogel microspheres containing one of the following branched ligands: 1) a dimer of the previously identified VEGFR2-derived VEGF-binding ligand^[8] that was generated by using a single lysine residue as the branch point ("dimer_c" - (EF_dA_dY_dL_dIDFNWEYPAS)₂KC); 2) a modified version of the dimer, with two additional lysines inserted into the ligand immediately before the branching point ("dimer_b" - (EF_dA_dY_dL_dIDFNWEYPAS)₂KKKC); and 3) a modified version of the dimer with one lysine added to each branch immediately after the branching point ("dimer_a" - (EF_dA_dY_dL_dIDFNWEYPASK)₂KC). We hypothesized that these branched ligands would bind VEGF with higher efficiency than the corresponding monomer, resulting in effective regulation of VEGF signaling.

VEGF sequestering by ligand-containing microspheres was strongly dependent on the ligand identity (Fig. 1a). Microspheres containing the monomer ligand bound $41 \pm 3.1\%$ ($K_d = 1$ nM) of the VEGF out of solution. In contrast, microspheres containing the dimer_c ligand showed significantly lower binding than those containing the corresponding monomer ligand in a serum-free PBS solution containing 4 ng/ml of VEGF. Dimer_c had a lower solubility than the corresponding monomer. In contrast, each of the dimer structures with lysine residues added at the branch point showed significantly higher binding than both the monomer and dimer_c spheres. The dimer_b spheres showed the highest binding affinity in serum-free conditions, removing $60 \pm 0.8\%$ ($K_d = 40$ pM) of VEGF from solution. Similarly, the dimer_a spheres bound $55 \pm 0.3\%$ ($K_d = 0.4$ nM) of the VEGF in solution. The additional lysine groups in dimer_a and dimer_b increased the solubility of the ligands relative to dimer_c, which likely played a role in the amount of peptide that was incorporated into the spheres (dimer_a = 2.1 ± 0.2 and dimer_b = 0.32 ± 0.2 μ g peptide/mg spheres). Microspheres containing a scrambled version of dimer_b (dimer_s - (DA_dPYNF_dEFAWKY_dISL_dE)₂KC) did not show statistically significant VEGF binding, demonstrating that VEGF binding was sequence specific.

Microspheres containing the modified dimers were subsequently tested over a range of VEGF concentrations and dimer_b consistently showed a higher VEGF binding efficiency than dimer_a (Fig. 1b). We next tested the microspheres in a medium containing 10% fetal bovine serum (FBS) in order to determine whether the enhanced VEGF binding would be

maintained in a serum-containing environment (Fig. 1c). As expected, each of the dimers bound less VEGF in serum-containing conditions when compared to serum-free conditions, with the reduction in binding being less for dimer_a microspheres than the dimer_b microspheres. Specifically, dimer_a microspheres bound $37 \pm 3\%$ of the VEGF in the serum containing solution while the dimer_b microspheres bound $29 \pm 4\%$ ($K_d = 0.1$ nM). In comparison, the monomer ligand in serum-containing medium bound only $22 \pm 4\%$

A human umbilical vein endothelial cell (HUVEC) proliferation assay was used to determine whether the hydrogel microspheres had the ability to impact VEGF-dependent cell behavior, as VEGF is a known HUVEC mitogen.^[9] Cells were cultured on the bottom of a 24 well plate and microspheres containing either the VEGF binding ligands or scrambled peptides were added to transwell inserts (0.2 μ m pores) above the cell culture. The cells were allowed to grow for three days in medium containing 10ng/ml of VEGF with 2% FBS, and cell number was measured using a Cell Titer Blue assay. An inverse relationship was found between the binding affinity of the spheres in the upper chamber and the proliferation rate of the HUVEC cells (Fig. 2a), consistent with the hypothesis that the spheres could sequester VEGF and downregulate VEGF mediated cell signaling.

Endothelial calcium signaling, a more immediate and direct measure of VEGF receptor activation,^[10] was also significantly reduced in cultures of uterine artery endodothelial cells (UAECs) containing dimer_a and dimer_b relative to conditions containing no spheres and spheres containing dimer_s (Fig. 2b). VEGF-induced calcium signaling was entirely knocked out in the presence of dimer_a, as calcium signaling dropped to $4 \pm 5\%$ of the control condition and the percent of responding UAECs was reduced to the same level as the VEGF-free condition.

We subsequently tested release of recombinant human VEGF from the spheres to demonstrate that the interaction with the ligand was reversible and determine if the VEGF was biologically active. Microspheres containing either dimer_a or dimer_b clearly demonstrated sustained VEGF release for over 30 days, and released significantly more VEGF and over a longer period of time relative to microspheres containing the monomer version of the peptide ligand (Fig. 3a). When incubated in a medium containing 10% FBS Dimer_a spheres released $48 \pm 5\%$ of the bound VEGF in 24 hours, while dimer_b ($72 \pm 2\%$) and monomer spheres ($85 \pm 10\%$) released a higher percentage of the bound growth factor. The observed release rates in serum are consistent with the relative affinities of the ligands that was observed in the serum-containing VEGF solution.

A HUVEC proliferation assay was used to confirm that the released VEGF was biologically active. Spheres containing dimer_a, dimer_b, and dimer_s were pre-loaded in serum-free buffer containing 25 ng/ml of VEGF, washed three times to remove unbound VEGF, and then introduced into the top of a transwell insert that was located above HUVECs. The cells were then incubated for three days and counted using a Cell Titer Blue assay. Both the dimer_a and dimer_b samples had higher cell counts than both the dimer_s samples and the control samples, which received no VEGF (Fig. 3b). Further, the cell counts in both the dimer_a and dimer_b samples were not significantly different from a sample that had been supplemented with 10 ng/ml of soluble VEGF.

In summary, using an approach that mimics the dimerization of VEGFR2, we developed functionalized hydrogel microspheres that efficiently bound VEGF with high specificity in both serum-containing and serum-free solutions. The structure of the branched peptide dimers dictated the VEGF binding efficiency, as the presence and location of charged residues near the branch point played a critical role in determining the VEGF binding. The resulting microspheres were capable of either down-regulating soluble VEGF signaling via

high affinity sequestering, or up-regulating VEGF signaling via binding and sustained release. Based on the broad prevalence of ligand dimerization and receptor dimerization during natural growth factor signalling, it is possible that this approach may be used for synthesis of other materials that use ligand branching to regulate specific growth factor signalling. These materials may also find applications in basic biology to locally knock out specific growth factor activity or deliver specific growth factors. This general approach may also be used to regulate the activity of autologous, cell-secreted growth factors, in a manner that is analogous to recent studies using proteoglycan sequestering,^[11–12] but with enhanced specificity.

Experimental

The peptide was synthesized on Tentagel S RAM resin (Anaspec) using a microwave peptide synthesizer (CEM). A lysine with Fmoc protecting groups on both amines was used to generate the two symmetrical branches of the dimers. Microspheres were formed in a water-in-water emulsion by adding a 18.6% w/w PEG dithiol (Laysan Bio)/6.4% w/w 4-arm PEG norbornene solution to a 40% w/w dextran 40K, resulting in a PEG rich dispersed phase. Dextran was weighed to make a 40% w/w solution in buffer (0.22 M KCl/10mM sodium phosphate/pH 8) that had been deoxygenated by purging with nitrogen through a diffusion stone for 10 minutes while stirring. PEG dithiol (3,400 MW) was weighed in a 1:1 functional group ratio with 4-arm PEG norbornene (20,000 MW), resulting in a 25 % w/w PEGDT/PEGNB solution in 0.5% w/w Irgacure 2959 (Ciba) photoinitiator solution. Spheres were generated using a water-in-water emulsion process utilizing thiol-ene chemistry to crosslink the PEG spheres and incorporate the cysteine functionalized peptides into the hydrogel network. Binding assays were performed in pH 7.4 PBS with 0.1% BSA. Eppendorf tubes containing the samples were incubated at 37 °C on an end-over-end mixer. I125-labeled VEGF was used to determine the concentration of VEGF in solution after binding. Samples were centrifuged at 8,000 RCF for five minutes. Four replicates were used for each binding assay while six replicates were used for HUVEC proliferation assays. Statistical differences were determined using a two-tail Student t-test.

Human umbilical vein endothelial cells (HUVECs) (Lonza) were seeded in gelatin-coated 24 well plates at 1.1×10^4 cells/well using Medium 199 (M199) supplemented with the EGM-2 bullet kit (Lonza). Cells were allowed to attach overnight. Ethanol-sterilized VBP or scramble peptide microspheres (5.4 mg per condition) were incubated with 10 ng/mL VEGF in M199/10% FBS (4.2 mL per condition) for 30 minutes. The microspheres were centrifuged and 0.7 mL of supernatant was added to each of 6 wells per condition. Transwell inserts (Corning) were placed into the wells in order to separate the microspheres from the cells. The microspheres were resuspended in the remaining M199/10% FBS and 0.2 mL added per transwell insert. For the No sphere condition, 0.7 mL of 10 ng/mL VEGF was added to each well and 0.2 mL of Medium 199/10% FBS was added to each transwell insert (n=6). Cell counts were determined after 72 hours using the CellTiter-Blue Cell Viability Assay (Promega).

For release experiments, ethanol-sterilized VBP or scramble peptide microspheres (5.4 mg per condition) were incubated with 25 ng/mL VEGF in M199/10% FBS (4.2 mL per condition) for 30 minutes. The microspheres were centrifuged and the supernatant discarded. The microspheres were then washed three times with sterile PBS solution. Transwell inserts were placed into the wells in order to separate the microspheres from the cells. The microspheres were resuspended in M199/10% FBS and 0.2 mL added per transwell insert. For the no microspheres condition, 0.2 mL of 45 ng/mL VEGF in M199/10% FBS was added to each transwell insert (n=6). Cell counts were determined after 72 hours using the CellTiter-Blue Cell Viability Assay.

Fura-2 Ca^{2+} imaging studies

UAEC were plated to low density (5–10% confluence) on 35-mm dishes with glass coverslip windows (Intracellular Imaging, Inc.) and grown to 70% confluence (4–5 days). Immediately before use, cells were loaded with Fura-2 AM for 45 minutes and rinsed three times in prewarmed (37 °C) Krebs buffer (125 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM KH_2PO_4 , 6 mM glucose, 2 mM CaCl_2 , and 25 mM HEPES, pH 7.4) before covering them in Krebs buffer (2 ml final volume) and incubating at room temperature for 30 minutes to allow ester hydrolysis. Fura-2 loading was verified by viewing at 380 nm UV excitation on a Nikon Diaphot inverted microscope (InCyt Im2, Intracellular Imaging, Inc.). About 60 cells were then preselected and video recordings commenced using alternate excitation at 340 and 380 nm at 1-second intervals and measuring emitted light using a digital camera. From the ratio of emission at 510 nm detected at the two excitation wavelengths, and by comparison to a standard curve established for the same settings using buffers of known free Ca^{2+} concentration, the $[\text{Ca}^{2+}]_i$ was calculated for individual cells in real time using InCyt Im2 software (Intracellular Imaging Inc.). Baseline $[\text{Ca}^{2+}]_i$ was recorded for 5 minutes before VEGF-165 (10ng/ml) treatment. Treatments were then recorded for 30 minutes from VEGF addition. Binding spheres (dimer_a, dimer_b, dimer_s) were pre-incubated for 30 minutes before baseline recording, allowing a minimum 35 minutes before VEGF-165 addition. Four dishes over a minimum of 2 days (separate platings) were used and Ca^{2+} responses were determined by $[\text{Ca}^{2+}]_i$ reaching twice basal levels and inverse readings for the 340 and 380nm excitations, as well as visual confirmation of obvious changes in $[\text{Ca}^{2+}]_i$. Details on the isolation and culturing of UAECs can be found in the Supporting Information file online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. VEGF sequestering by peptide-functionalized PEG microspheres

a) Sequestering in a 4 ng/ml VEGF solution in PBS; a representative picture of PEG microspheres is included (inset). b) VEGF binding by PEG microspheres containing lysine modified branched dimers in solutions with varying concentrations of VEGF. c) VEGF binding in a 4 ng/ml VEGF solution containing 10% fetal bovine serum. * ($p < 0.05$) and # ($p < 0.01$) indicate statistically significant difference in binding when compared to microspheres containing the monomeric ligand.

Figure 2. VEGF sequestration by microspheres in cell culture

a) Spheres incubated with HUVEC cells with VEGF for 72 hours. * ($p < 0.05$) and # ($p < 0.01$) indicate statistically significant difference in cell count in comparison to 10 ng/ml VEGF condition. b) UAEC calcium signalling response to 10 ng/ml VEGF in the presence of microspheres. * ($p < 0.05$) indicates statistically significant reduction in number of responding cells in comparison to Control.

Figure 3. Release of VEGF from functionalized microspheres

a) Release over time from microspheres pre-incubated in 40 ng/ml VEGF solution. b) HUVEC cells grown with soluble VEGF supplement or microspheres pre-incubated in VEGF.