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Surface Density of Vascular Endothelial Growth Factor Modulates Endothelial Proliferation and Differentiation

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ABSTRACT

Therapeutic strategies aim to regulate vasculature either by encouraging vessel growth for tissue engineering or inhibiting vascularization around a tumor. Vascular endothelial growth factor (VEGF) is essential to these processes, and there are several strategies that manipulate VEGF signaling. Here we develop a method to control the surface density of VEGF, which is covalently attached to tissue culture polystyrene (TCPS), and explore cellular responses to surfaces with varying VEGF densities. We show that the crosslinker reduces but does not eliminate the biological activity of soluble VEGF as measured by endothelial proliferation. However, endothelial cells cultured on surfaces of covalently bound VEGF did not proliferate in response to surface cues. Interestingly, compared to cells incubated with soluble VEGF (10 ng/ml) and cultured on TCPS, lower cell proliferation was observed when endothelial cells were cultured on high VEGF surface densities (5.9 ng/cm^2), whereas higher cell proliferation occurred when cells were cultured on low surface densities (0.04 ng/cm^2). High density surfaces (5.9 ng/cm^2) also acted in synergy with an inhibitor of VEGF receptors to further suppress endothelial cell proliferation. We also examined the effect of VEGF surfaces on endothelial differentiation of mesenchymal stem cells. No effect was observed when cells were cultured on VEGF surfaces; however, the VEGF surfaces acted in synergy with an inhibitor of VEGF receptors to decrease the ability of differentiated cells to form vascular networks. Together, these results suggest that surface density of bound VEGF can be used to modulate cell behavior and inhibit an angiogenic response. J. Cell. Biochem. 115: 111–120, 2014.

KEY WORDS: VEGF INHIBITORS; MESENCHYMAL STEM CELLS; TISSUE ENGINEERING; VASCULARIZATION; ENDOTHELIAL CELLS; ANGIOGENESIS

The VEGF family consists of five glycoproteins: VEGF-A, -B, -C, -D, and placental growth factor. These cytokines promote and regulate vasculogenesis and angiogenesis by stimulating cell survival, proliferation, and migration. Cells proliferate in response to VEGF concentrations and migrate in response to a VEGF gradient [Gerhardt et al., 2003]. Tube morphogenesis is guided by hematopoietic cytokines, but priming by VEGF is required for the process to occur [Stratman et al., 2011]. The extent of network branching is determined by the spatial presentation of heparin-bound VEGF [Ruhrberg et al., 2002].

The most well-studied member of the family, VEGF-A (and its isoforms of 121, 165, 189, and 206 amino acids), binds to three VEGF receptors: VEGFR1, VEGFR2, and VEGFR3 [Ferrara et al., 2003]. VEGF-A is a disulfide-linked homodimer with two receptor binding sites [Muller et al., 1997]. VEGF receptors can bind to each site independently, and receptor dimerization occurs once receptors have bound both sites. Upon dimerization, autophosphorylation and signal transduction occur [Schlessinger, 2000]. Manipulating this signaling process could provide a way to control vascular development. Preventing vascular growth is of interest to cancer researchers because tumors cannot grow beyond 1–2 cm^3 without recruiting vascular support [Folkman, 1990]. Conversely, stimulating vascular growth is of interest to tissue engineers because, without vascular support, the length scale of engineered constructs is limited by diffusion to 150–200 μm [Colton, 1995].

Cancer research on blocking vasculature development has focused on silencing or preventing signals from being transduced. Strategies for inhibiting development have been devised for all parts of the signaling process. For example, monoclonal antibodies [Ferrara et al., 2004], fragments of antibodies [Chen et al., 1999; Gragoudas et al., 2004], and engineered analogs of VEGF receptors

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[Economides et al., 2003] have been used to prevent soluble VEGF from binding to VEGF receptors. VEGF receptors have been blocked by heterodimers of VEGF [Fuh et al., 1998; Siemeister et al., 1998; Boesen et al., 2002] and peptide fragments of VEGF [Soker et al., 1997; Koepsel et al., 2012]. Phosphorylation of the receptors has also been prevented by several classes of small molecules designed to antagonize the adenosine triphosphate (ATP) binding pocket of VEGF receptors [Gotink and Verheul, 2010].

Attempts to encourage vascular development for tissue engineering applications have been made largely by increasing VEGF signaling. Approaches have encompassed gene delivery [Tao et al., 2006; Lin et al., 2008], soluble peptides [D'Andrea et al., 2005], soluble VEGF [Ennett et al., 2006; Geng et al., 2011], bound peptides [Lee et al., 2010], bound VEGF, and cleavable VEGF [Zisch et al., 2003]. Recently, it has been shown that VEGF receptor phosphorylation varies depending on whether VEGF is soluble or bound to a surface [Chen et al., 2010]. Studies have attached VEGF through covalent bonds or electrostatic interactions to two- [Backer et al., 2006; Alberti et al., 2008; Shin et al., 2012] and three-dimensional [Zisch et al., 2001; Koch et al., 2006; Leslie-Barbick et al., 2009; Miyagi et al., 2011; Porter et al., 2011] material surfaces. The binding of VEGF has either been randomly oriented through crosslinking primary amines [Koch et al., 2006; Leslie-Barbick et al., 2009; Chiang et al., 2010; Chiu and Radisic, 2010; Poh et al., 2010; Miyagi et al., 2011; Porter et al., 2011; Hu et al., 2012; Shin et al., 2012] or been oriented through cysteine tags [Backer et al., 2006], binding to heparin [Ohyama et al., 2004; Anderson et al., 2009; Singh et al., 2011; Ye et al., 2012] or heparin-like functional groups [Crombez et al., 2005; Wang et al., 2008], and direct binding to collagen [Chen et al., 2010]. Responses to bound VEGF have been quantified by examining phosphorylation of VEGF R2 [Anderson et al., 2009; Chen et al., 2010]; endothelial cell clustering [Backer et al., 2006]; endothelial proliferation [Tao et al., 2006]; increased vascular ingrowth in vivo [Miyagi et al., 2011]; increased de novo network formation [Leslie-Barbick et al., 2011]; and endothelial differentiation of endothelial progenitor cells [Ehrbar et al., 2005], mesenchymal stem cells (MSCs) [Poh et al., 2010], or embryonic stem cells [Chiang et al., 2010].

In the present work, we varied the surface density of covalently bound VEGF and investigated its effect on proliferation of primary endothelial cells and endothelial differentiation of MSCs. To attach VEGF to TCPS, we used a bifunctional crosslinker that reacts with primary amines in VEGF and photocrosslinks to TCPS (Fig. 1). The results indicated that the crosslinker reduced the bioactivity of VEGF but did not completely eliminate a biological response. We discovered that high surface densities of VEGF inhibited proliferative cues from soluble VEGF. Likewise, VEGF surfaces acted in synergy with an inhibitor of VEGF receptors (AZD2171, Cediranib/Recentin) to prevent differentiated cells from forming networks on ECMatrix. These results indicate that surface density of VEGF affects cell response and that at particular surface densities, randomly oriented VEGF can inhibit an angiogenic response. Extending our understanding of VEGF presentation could ultimately lead to new strategies to promote or inhibit vascular development.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were obtained from Sigma–Aldrich (St. Louis, MO).

VEGF MODIFICATION

A 10^{-6} M solution of glycosylated recombinant human VEGF (R&D Systems, Minneapolis, MN, courtesy of the National Cancer Institute Preclinical Repository) was reacted in the dark with 50-fold excess of *N*-sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (sulfo-SANPAH, Pierce Biotechnology, Rockford, IL) in 1× HEPES-buffered saline (140 mM NaCl, 1.5 mM $Na_2HPO_4{\cdot}2H_2O$, 50 mM HEPES, pH 7.4 with NaOH) for 6 h at room temperature. The reaction was quenched by diluting the reaction mixture with 1 M Tris buffer (J.T. Baker, Phillipsburg, NJ), pH 7.5, to a final concentration of 0.2 M Tris.

Bovine serum albumin (BSA) was used to confirm reaction conditions (Supplementary Fig. 1A). BSA was reacted with sulfo-SANPAH, and excess sulfo-SANPAH was removed via dialysis (Spectra/Por Dialysis Membrane, MWCO 25,000, Spectrum Labs, Rancho Dominguez, CA) against water (Milli-Q Gradient, Millipore, Danvers, MA). The resulting absorption spectrum was read on a SpectraMax M2^e (Molecular Devices, Downingtown, PA) plate reader using UV-Star 96-well half-area plates (Greiner Bio-One, Monroe, NC).

CONJUGATION OF VEGF TO A SURFACE

Conjugation was performed using a method similar to what has previously been reported for the attachment of different growth factors [Stefonek and Masters, 2007; Stefonek-Puccinelli and Masters, 2008]. The quenched crosslinking reaction was diluted 350- to 160,000-fold, and 128 μl of solution per cm^2 was dispensed onto TCPS plates (BD Biosciences, San Jose, CA). Plates were dried overnight at 40°C in an oven containing desiccant and were then exposed to 21.7 mW/cm^2 of 365 nm light (Blak-Ray B-100AP/R, Ultra Violet Products, Upland, CA) for 5 min. To confirm the complete reaction of the 4′-azido-2′-nitrophenylamino group in sulfo-SANPAH, we used BSA as a model protein and examined the absorption spectrum as a function of reaction time (Supplementary Fig. 1B,C).

Previous studies used acidic solutions to remove VEGF physically adsorbed to a surface [Chiu and Radisic, 2010], and we also used acidic solutions to remove VEGF not covalently attached to the surface (Supplementary Fig. 2). Surfaces were rinsed in sterile phosphate buffered saline (PBS) for 2 additional days before use. An enzyme-linked immunosorbent assay (ELISA) (Supplementary Fig. 2A) confirmed that acid treatment resulted in lower surface densities of active VEGF. A bicinchoninic acid assay (QuantiPro BCA Assay Kit) confirmed that acid treatment removed adsorbed VEGF and did not simply denature the bound protein (Supplementary Fig. 2B).

CHARACTERIZING VEGF SURFACE DENSITIES WITH ELISA

An ELISA was used to characterize the density of VEGF bound to the surface. First, a standard curve using soluble VEGF was determined. A 10 μg/ml solution of capture antibody (MAB293, R&D Systems) was

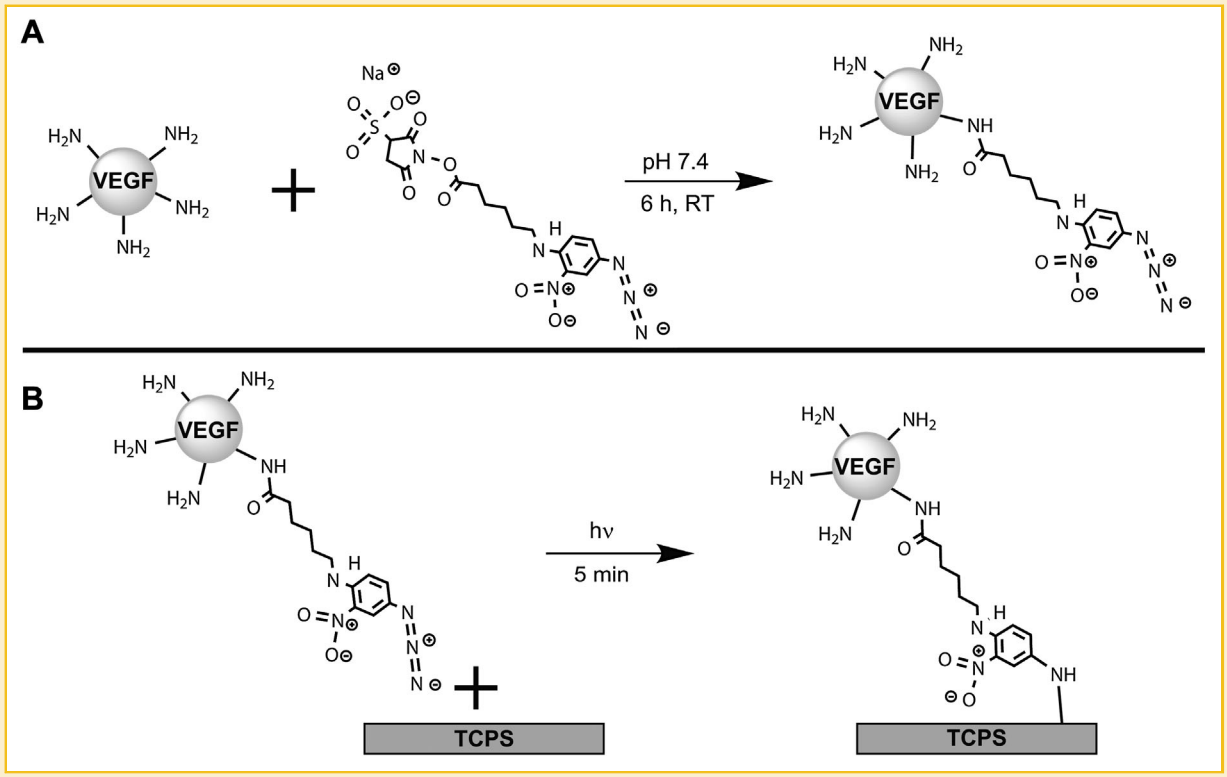

Fig. 1. Schematic of (A) the conjugation of sulfo-SANPAH to VEGF and (B) the reaction of the VEGF-sulfo-SANPAH complex with the surface. A: The sulfo-*N*-hydroxysuccinimide ester group in sulfo-SANPAH reacts with primary amines in VEGF. There are 10 primary amines on the surface of VEGF, and five of them are represented in the schematic. B: Exposure to light causes the azide to form N_2 and a nitrene. The nitrene then reacts with functional groups on the TCPS surface.

adsorbed onto high-binding 96-well plates (MaxiSorp ImmunoModule Plate, Nunc Nalgene, Rochester, NY) overnight (>16 h) at room temperature. Surfaces were then blocked with BSA. A dilution series of VEGF was bound to capture antibodies. A 0.25 μg/ml solution of biotinylated detection antibody (BAF293, R&D Systems) was added to bind VEGF, and horseradish peroxidase conjugated to streptavidin (Streptavidin Horseradish Peroxidase, Trevigen, Gaithersburg, MD) was added to bind the detection antibodies. The enzyme complex was incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Danvers, MA) for 10–15 min. Then, 1 M hydrochloric acid was added (50 μL of acid per 100 μl of TMB), absorbance readings were taken at 450 nm, and background readings were taken at 540 nm. To quantify VEGF surface density, the standard ELISA protocol was modified to detect covalently bound VEGF [Anderson et al., 2009; Chiang et al., 2010]. Briefly, modified surfaces were blocked with BSA and then incubated with detection antibody and horseradish peroxidase. The absorbance readings of the surfaces and soluble standards were then compared.

CELL CULTURE

Cells were cultured at 37°C with 5% CO_2 in a humidified environment. Human umbilical vein endothelial cells (HUVECs) and human MSCs were purchased from Lonza (Walkersville, MD). HUVECs were maintained in endothelial basal medium (EBM-2) supplemented with growth factors (EGM-2, Lonza) and used between passages 3 and 8. Human MSCs were used at passage 3 and were maintained in a maintenance medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, catalog # 14-501F, Lonza), 10 ng/ml fibroblast growth factor (R&D Systems, courtesy of the National Cancer Institute Preclinical Repository), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were subcultured at 80–90% confluence using 0.05% Trypsin–EDTA (Gibco, Grand Island, NY).

LIVE/DEAD CYTOTOXICITY ASSAY

HUVECs were seeded at 6,250 cells/cm^2 on VEGF-modified surfaces and cultured for 3 days in EGM-2. Cells were incubated in PBS containing 0.5 nM calcein AM, 0.75 mM ethidium homodimer-1 (LIVE/DEAD kit, Life Technologies, Carlsbad, CA), 0.90 mM calcium chloride, and 0.49 mM magnesium chloride for 45 min at 37°C. After rinsing, cells were imaged using a Nikon Ti-E microscope (Nikon, Melville, NY). Calcein AM was imaged using a fluorescein isothiocyanate (FITC) filter cube, and ethidium homodimer-1 was imaged using a tetramethyl rhodamine isothiocyanate (TRITC) filter cube. The ethidium homodimer-1 dye, which stains dead cells, was validated with a control population treated with 95% ethanol for 30 min. Cell numbers were quantified using NIS-Elements software (Nikon). At least 1,000 cells per population (n = 3) were examined.

WST-1 PROLIFERATION ASSAY

The effect of VEGF modified with sulfo-SANPAH (VEGF-SS) and surface-bound VEGF on cell proliferation was examined with two

distinct protocols. For studies with soluble VEGF-SS, HUVECs at 80% confluence were serum-starved for 24 h in DMEM containing 0.1% FBS. After 24 h, VEGF-SS or VEGF was added to the low serum medium, and cells were cultured for an additional 24 h. To assess metabolic activity, 10 μl of WST-1 (G Biosciences, St. Louis, MO) was added to 100 μl medium and incubated for 4 h at 37°C. Absorbance of the medium was measured at 450 nm and corrected by a background reading of medium without cells. All experimental absorbance readings were within the linear range of the assay as determined by a standard curve.

To assess modified surfaces, HUVECs at 80% confluence were grown in incomplete EGM-2 (EBM-2 with 2% serum but no growth factors added) for 24 h. Cells were lifted with 0.05% Trypsin–EDTA, seeded onto surfaces at 6,250 cells/cm^2, and cultured for the specified time period in incomplete EGM-2. The positive control group consisted of adding 10 μg/ml of soluble VEGF to incomplete EGM-2. For the treatment groups containing AZD2171 (Selleck Chemicals, Houston, TX), which inhibits VEGF receptors, AZD2171 was resuspended in dimethyl sulfoxide at 100 μM and diluted 1:1,000 in medium. Medium was changed daily, and fresh supplements were used. Metabolic activity was assessed with WST-1 as described above.

ENDOTHELIAL DIFFERENTIATION

Human MSCs were seeded onto surfaces at 3,750 cells/cm^2 in endothelial differentiation medium consisting of EGM-2 with all aliquots added except for that of VEGF. Medium was changed every other day. A negative control group consisted of cells seeded on TCPS and cultured in endothelial differentiation medium. A positive control group consisted of cells seeded on TCPS in endothelial differentiation medium supplemented with 50 or 100 ng/ml of soluble VEGF. Treatment groups with inhibitor contained 0.1 μM AZD2171. After treatment for the specified time, cells were trypsinized and resuspended for immunofluorescence studies, acetylated low-density lipoprotein (ac-LDL) uptake studies, or in vitro angiogenesis assays.

IMMUNOFLUORESCENCE

After cells were treated with differentiation medium for 0, 1, 2, and 3 weeks, they were trypsinized, pelleted, resuspended in EGM-2, and seeded at a density of 10,000 cells/cm^2 on glass coverslips. Cells were incubated for 4 h at 37°C in EGM-2 to allow the cells to adhere and spread. Cells were rinsed twice with PBS, fixed in a 4% paraformaldehyde solution for 25 min, and rinsed twice with PBS. When staining for von Willebrand factor (vWF), Ephrin-B1, or Ephrin type-B receptor 4 (EphB4), samples were permeabilized with a 1% Triton X-100 solution for 5 min. Nonspecific binding was blocked by treating cells with a 5% BSA solution for 1 h. Primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in blocking solution and incubated with cells. Antibodies targeting vascular endothelial cadherin (VE-Cadherin) (sc-9989, diluted 1:100), VEGF R2 (sc-6251, diluted 1:50), vWF (sc-53666, diluted 1:50), and EphB4 (sc-130062, diluted 1:25) were incubated for 1 h at room temperature. Antibodies against Ephrin-B1 (sc-20723, diluted 1:75) were incubated overnight at 4°C. Cells were rinsed with PBS and incubated for 1 h with Alexa Fluor 488-tagged secondary antibody (A11001, Invitrogen, Carlsbad, CA) diluted 1:1,000 in blocking solution. Cells were rinsed in PBS and incubated for 30 min at room temperature in the nuclear dye, DRAQ5 (Biostatus Limited, Shepshed, UK), diluted 1:250 in PBS. Coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA) and stored in the dark at 4°C until imaging.

Cells were imaged with a confocal microscope (Nikon Ti-E, Eclipse C-1 Plus) and a 20× objective. All samples were imaged on the same day with identical laser intensity and gain settings. Nikon NIS-Elements AR software (version 3.2) was used to analyze fluorescence images. Individual cells were selected as regions of interest (ROI) by setting threshold levels on both pixel intensity and cell area. The mean intensity per cell was quantified by the software (using the ROI Stats feature) as the mean of all pixel values within a cell. The average and standard deviation were obtained by examining the mean intensities of three populations for each treatment. At least 100 cells were imaged for each cell population.

ACETYLATED-LDL (AC-LDL) UPTAKE

After cells were treated with differentiation medium for 0, 2, and 3 weeks, they were trypsinized, pelleted, resuspended in endothelial differentiation medium, and seeded on glass coverslips at a density of 10,000 cells/cm^2. Cells were incubated for 4 h at 37°C to allow the cells to adhere and spread. The ability of treated cells to uptake ac-LDL was assessed with ac-LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-ac-LDL, Biomedical Technologies, Stoughton, MA). Cells were incubated in the dark for 4 h in endothelial differentiation medium containing 10 μg/ml DiI-ac-LDL. Cells were rinsed three times with warm PBS and mounted onto coverslips in a 1:1 solution of glycerol and PBS. Cells were imaged with a Nikon Ti-E using a differential interference contrast (DIC) 20× objective, and DiI-ac-LDL uptake was assessed by examining DiI fluorescence with a TRITC filter cube. Cells positive for Dil-ac-LDL uptake were determined by setting the pixel threshold to 1.5 times the background levels. The total cell numbers were determined manually using the DIC images.

ANGIOGENIC RESPONSE

To assess angiogenic response, we used an in vitro angiogenesis kit (ECM625, Millipore, Billerinca, MA) derived from the basement proteins secreted by Engelbreth Holm–Swarm mouse tumor cells. The gel, referred to as ECMatrix, was prepared according to the manufacturer's instructions. Briefly, 50 μl of the gel solution was pipetted onto the bottom of each well in a 96-well plate. The plate was incubated for 1 h at 37°C. Cells treated with differentiation medium for 1 week were seeded on the gel at 10,000 cells/well. After 5 h in MSC maintenance medium, cells were imaged on a Nikon Ti-E inverted microscope using a 4× objective. Four images (each 1.95 mm^2) from a single well were combined to produce a representative sample area. Three wells were examined for each treatment. A blinded analyst manually traced the network length and counted the number of branch points in each image using the Nikon NIS-Elements AR software.

STATISTICS

Statistical differences between VEGF surface densities were determined using a two-tailed, unpaired *t*-test assuming equal variance. A one-way analysis of variance (ANOVA) was used to examine the data

from the proliferation and angiogenesis assays. Tukey's post hoc analysis was used to organize treatments into statistically equivalent ($P > 0.05$) subgroups (denoted by letters). For immunofluorescence and ac-LDL uptake studies, a two-way ANOVA was performed, and Tukey's post hoc analysis was performed to arrange statistically equivalent ($P > 0.05$) subgroups of treatments and time points (denoted by letters). For immunofluorescence and ac-LDL studies, a one-way ANOVA was used to compare treated groups at each time point to proliferating MSCs at week 0. Dunnett's post hoc analysis was used to determine whether treated populations were statistically different ($P < 0.05$) from proliferating MSCs (denoted by asterisks). The assumption of equal variance was justified by the Modified Levene's (Brown–Forsythe) test ($P > 0.05$). The assumption of normality was justified by the Komogorov–Smirnov, Cramer–von Mises, and Anderson–Darling goodness-of-fit tests ($P > 0.05$). Analysis was performed in SAS version 9.3 (SAS Institute, Cary, NC). Error bars represent 1 SD.

RESULTS

MODIFIED VEGF RETAINED BIOLOGICAL ACTIVITY IN SOLUTION

One of the primary amines on VEGF is located in an active receptor site [Muller et al., 1997]. Therefore, it is possible that modification with sulfo-SANPAH could sterically inhibit receptor binding and render VEGF-SS inactive. To assess the biological activity of VEGF-SS, a proliferation assay was performed on HUVECs exposed to VEGF-SS or VEGF (Fig. 2). As expected, cells cultured with VEGF had an increase in cell number compared to cells cultured without VEGF. Furthermore, HUVECs exhibited a dose-dependent increase in response to VEGF-SS. However, VEGF-SS had statistically lower biological activity compared to VEGF at the same concentration, and twice as much VEGF-SS was needed to observe the same proliferative response elicited by VEGF.

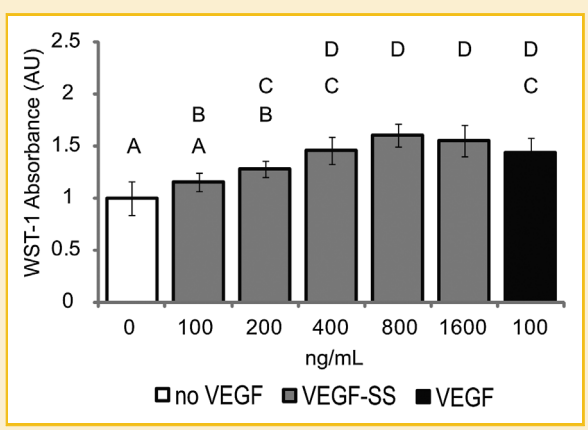

Fig. 2. HUVECs proliferated in response to soluble VEGF modified with sulfo-SANPAH (VEGF-SS). A colorimetric WST-1 assay was used, and absorbance readings positively correlated with cell number. Cells were incubated for 24 h in medium containing low serum (0.1%) and no VEGF (white), soluble VEGF-SS at varying concentrations (gray), or soluble VEGF (black) ($n = 5$). Letters indicate statistically equivalent Tukey groups ($P > 0.05$). Error bars indicate 1 SD.

CHARACTERIZATION OF THE VEGF SURFACES

ELISAs were used to quantify the surface density of VEGF and to detect any VEGF released by the surface. To vary the surface density of VEGF, a halving dilution series of VEGF-SS was dried on the surface and resulted in VEGF surface densities differing by a factor of approximately 2 (Fig. 3A). Surface densities varied from a low of 0.04 ng VEGF/cm^2 to a high of 5.9 ng VEGF/cm^2 (surfaces are hereafter referred to as s-X, where X represents the surface density in ng VEGF/cm^2). The difference between each surface and the one preceding it was found to be statistically significant ($P < 0.05$) when using a *t*-test.

VEGF surfaces were incubated in EBM-2 at 37°C for 1 week to examine whether VEGF was released from the surface over time. In three independent experiments, the VEGF density readings of surfaces were statistically equivalent when they were incubated in EBM-2 or stored dry for 7 days (Fig. 3B). No VEGF was detected in the supernatant (data not shown). Taken together, these results indicate that VEGF is covalently bound to the surface and is stable and unaffected by medium changes over a 7-day period.

HUVECs were seeded onto surfaces modified with VEGF to test for cytocompatibility. The micrograph in Figure 3C shows that cells adhered and spread on the VEGF surface (s-5.9). After 3 days, the cell viability was 99.2 ± 0.9%. Thus, the VEGF surface did not prevent cell attachment and spreading and displayed no cytotoxic effects.

SURFACE-BOUND VEGF DID NOT STIMULATE ENDOTHELIAL CELL PROLIFERATION

To assess the ability of surface-bound VEGF to stimulate endothelial cell proliferation, HUVECs were seeded on surfaces of varying densities and cultured for 5, 7, or 9 days in incomplete EGM-2, which lacks supplemental growth factors (Fig. 4). No proliferation or apoptosis was observed for negative control cells, which were cultured on untreated TCPS, over the 9-day period (data not shown). At all three-time points, cell proliferation on VEGF surfaces was statistically similar to the negative control. In contrast, the positive control cells, which were seeded on TCPS and cultured in medium containing 10 ng/ml soluble VEGF, proliferated through the duration of the experiment (data not shown). At each time point, the positive control group had statistically higher WST-1 activity than either the negative control or cells cultured on the VEGF surface.

SURFACE-BOUND VEGF INHIBITED THE EFFECT OF SOLUBLE VEGF

To better understand the effect of the VEGF surfaces on the VEGF signaling pathway, cells on VEGF surfaces were incubated in medium supplemented with 10 ng/ml soluble VEGF; 0.1 μM AZD2171, which inhibits VEGF receptors; both soluble VEGF and AZD2171; or neither (negative control) (Fig. 5). When cultured in negative control medium, all surface densities of VEGF resulted in statistically equivalent WST-1 activity. Proliferative responses on 4 of the 6 surface densities (s-0.2, s-0.5, s-1.2, and s-2.8) were statistically equivalent to cells cultured on TCPS. These results are in general agreement with the results shown in Figure 4B.

Cells on TCPS had a statistically significant 1.5-fold increase in WST-1 response when cultured in medium containing soluble VEGF compared to negative control medium. Cells seeded on surface

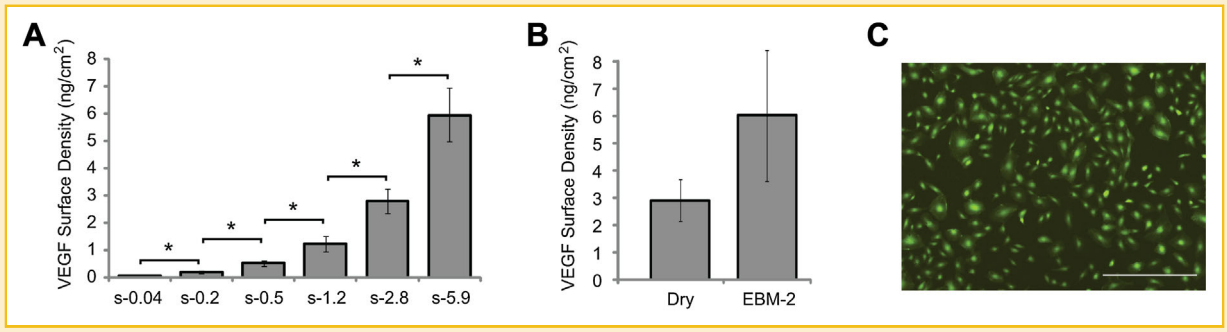

Fig. 3. VEGF-SS was successfully attached to TCPS. A: The surface density of bound VEGF can be varied. Six surface densities were created by varying the amount of VEGF onTCPS ($n = 6$). (*) Indicates all surface densities were found to be statistically different from each other when compared with *t*-tests ($P < 0.05$). Error bars represent 1 SD. B: Covalently bound VEGF does not desorb into the medium over 7 days of incubation in EBM-2 with medium changes every other day. The VEGF surface density was found to be statistically equivalent to control surfaces not incubated in EGM-2 (kept dry for 7 days) when compared using a *t*-test ($n = 4$). No soluble VEGF was detected in the medium (data not shown). Error bars represent 1 SD. C: The VEGF-modified surface is cytocompatible with HUVECs after 3 days of culture. HUVECs were seeded on surfaces with a measured VEGF density of 5.9 ng/cm^2. Calcein AM stained live cells (green), and ethidium homodimer-1 stained dead cells (red). At least 1,000 cells per well ($n = 3$) were analyzed. Quantitative analysis indicated that 99.2 $\pm$ 0.9% of all cells were viable. Scale bar represents 500 μm.

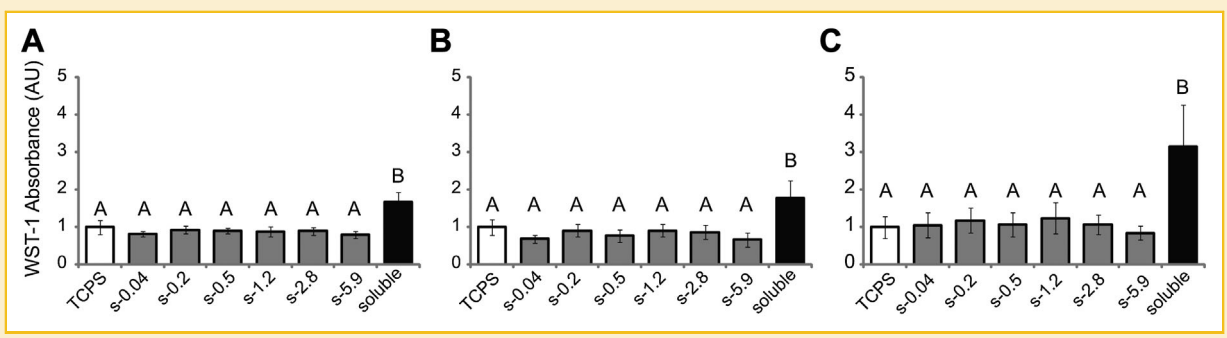

Fig. 4. HUVECs did not proliferate on surfaces modified with VEGF. WST-1 assays were performed at (A) 5 days, (B) 7 days, and (C) 9 days. At each time point, the metabolic activity of the negative control (cells cultured on TCPS, white) was statistically equivalent to the metabolic activity of cells cultured on surfaces with densities varying between 0.04 and 5.9 ng/cm^2 (shown in gray, defined as s-X where X denotes the surface density in ng VEGF/cm^2). The metabolic activity of positive control cells (cells treated with 10 ng/ml soluble VEGF, black) was statistically higher than the negative control or cells grown on the VEGF surfaces. For each time point, letters indicate statistically similar groups as determined from Tukey's post hoc analysis ($P > 0.05$) of a one-way ANOVA. Error bars indicate 1 SD.

densities between 0.04 and 2.8 ng/cm^2 had WST-1 responses statistically higher than or equal to cells on TCPS. Interestingly, cells seeded on s-5.9 had the lowest WST-1 response of all cells treated with soluble VEGF. In fact, these cells had a WST-1 response statistically equivalent to cells on TCPS in negative control medium. In other words, soluble VEGF did not have a positive effect on proliferation when cells were seeded on s-5.9.

In groups where AZD2171 was added to the medium, no effect on proliferation was observed when comparing between VEGF surfaces of varying density. However, cells on s-5.9 had a WST-1 response statistically lower than cells seeded on TCPS. Interestingly, when comparing surfaces cultured with inhibitor versus negative control medium, the surface with the highest VEGF density was the only surface that statistically lowered cell proliferation in the presence of inhibitor.

When the medium was supplemented with both AZD2171 and soluble VEGF, the proliferative effect of soluble VEGF was negated. Furthermore, all surface densities of VEGF were statistically equivalent to each other. In addition, cells seeded on s-0.04 had a statistically lower proliferative response compared to cells seeded on TCPS. When comparing proliferation of cells cultured with both soluble VEGF and AZD2171 to cells cultured with just AZD2171, no statistically significant differences were observed for any surface density of VEGF.

SURFACE-BOUND VEGF WORKS IN SYNERGY WITH A VEGF RECEPTOR INHIBITOR

MSCs were differentiated into endothelial cells on VEGF surfaces and were compared to cells differentiated on TCPS in the presence of 50 or 100 ng/ml soluble VEGF. Over 3 weeks, no differences in protein expression of VE-Cadherin, vWF, VEGF R2, EphB4, or Ephrin-B1 were observed when compared to TCPS controls (Supplementary Fig. 3A–E). No difference in ac-LDL uptake was observed between cells differentiated on TCPS or surfaces with bound VEGF (Supplementary Fig. 3F).

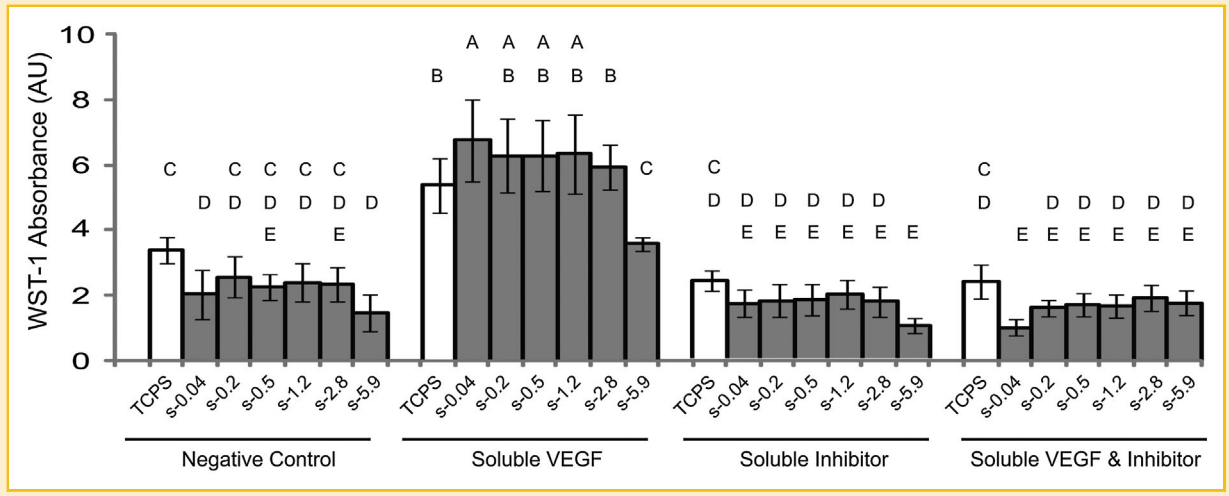

Fig. 5. VEGF surfaces affected HUVEC proliferation in concert with soluble signals. HUVEC proliferation was examined after 7 days of culture in EBM-2 containing 2% serum and 10 ng/ml soluble VEGF, 0.1 μM AZD2171 (inhibitor of VEGF receptors), or a combination of both ($n = 6$). Letters indicate statistically similar groups as determined from Tukey's post hoc analysis ($P > 0.05$) of a one-way ANOVA. Error bars indicate 1 SD.

MSCs were differentiated for 1 week in the presence or absence of AZD2171 and were then seeded onto ECMatrix for 5 h. Micrographs in Figure 6 A show the characteristic cell response to ECMatrix. Most treatment groups appeared to form rudimentary networks, but cells differentiated on s-18.6 in the presence of AZD2171 formed little to no networks. Quantification of networks revealed that cells differentiated on s-18.6 in the presence of the inhibitor formed networks with statistically fewer branch points compared to negative control cells (cells grown on TCPS) or positive control cells (cells grown on TCPS and cultured with soluble VEGF).

DISCUSSION

We explored the effect of VEGF surface density on cellular responses. VEGF was covalently attached to surfaces at surface densities ranging from 0.04 to 18.6 ng/cm^2. VEGF receptor phosphorylation is known to occur at VEGF surface densities as low as 0.12 ng/cm^2 [Chiang et al., 2010]. However, our VEGF surface densities are on the lower end of the range previously used to induce a cellular response in two dimensions (0.9–722 ng/cm^2) [Backer et al., 2006; Leslie-Barbick et al., 2009; Chiang et al., 2010; Poh et al., 2010; Hu et al., 2012; Ye et al., 2012].

Neither endothelial cells nor MSCs seeded on surfaces of varying VEGF densities exhibited a biological response in the absence of medium supplements. Studies by Backer et al. [2006] found that endothelial proliferation could be weakly stimulated with VEGF surface densities as low as 2.8 ng/cm^2 but that cells were optimally stimulated at densities of 28 ng/cm^2 VEGF. One difference between our studies and theirs is that Backer et al. [2006] used VEGF that was oriented with a genetically engineered tag on the N-terminus. Other studies using VEGF surface densities of 0.1–91 ng/cm^2 observed that endothelial cells had a proliferative response to the VEGF surfaces [Ehrbar et al., 2005; Backer et al., 2006; Anderson et al., 2009; Poh et al., 2010; Shin et al., 2012]. However, all studies with surface densities below 26 ng/cm^2 utilized oriented VEGF [Backer et al., 2006; Anderson et al., 2009]. Our studies demonstrated that endothelial cells seeded onto surfaces modified with VEGF-SS did not exhibit a proliferative response. This lack of proliferative response could be because: the crosslinker attaches to a lysine residue(s) that interferes with VEGF activity; our method for attaching VEGF results in random orientation on the surface, which interferes with VEGF activity; or the surface density required for a proliferative response is higher than the range we tested.

The endothelial differentiation of MSCs was not inhibited by VEGF surfaces. No change in protein expression, ability to internalize ac-LDL, or angiogenic response to ECMatrix was observed when compared to TCPS controls (Fig. 6 and Supplementary Fig. 3). Levels of immunofluorescent staining and angiogenic response of our differentiated cells were similar to observations by Poh et al. [2010] in which MSCs were differentiated on VEGF covalently bound to titanium through polydopamine (VEGF surface density of 26 ng/cm^2). However, cells on our control surface (TCPS) had statistically similar protein expression and angiogenic response compared to cells on s-18.6, whereas cells on the control surface in Poh et al. [2010] (titanium) resulted in different protein expression and angiogenic behavior compared to cells on VEGF-modified titanium surfaces. This discrepancy implies that the underlying substrate can also influence the differentiation.

Seeding cells on s-0.04 increased the proliferative effect of soluble VEGF; however, seeding cells on s-5.9 inhibited the proliferative effect of soluble VEGF. A study by Koepsel et al. [2012] also found that seeding cells on surfaces with a VEGF-mimicking peptide decreased the proliferative effect of soluble VEGF. The authors proposed that the rigidity of the covalent bond between the peptide and surface caused the receptors to become "pinned," which prevented dimerization and phosphorylation [Koepsel et al., 2012]. Thus, the covalently bound peptide acted as an antagonist. Together, these results suggest a potential new method for inhibiting soluble

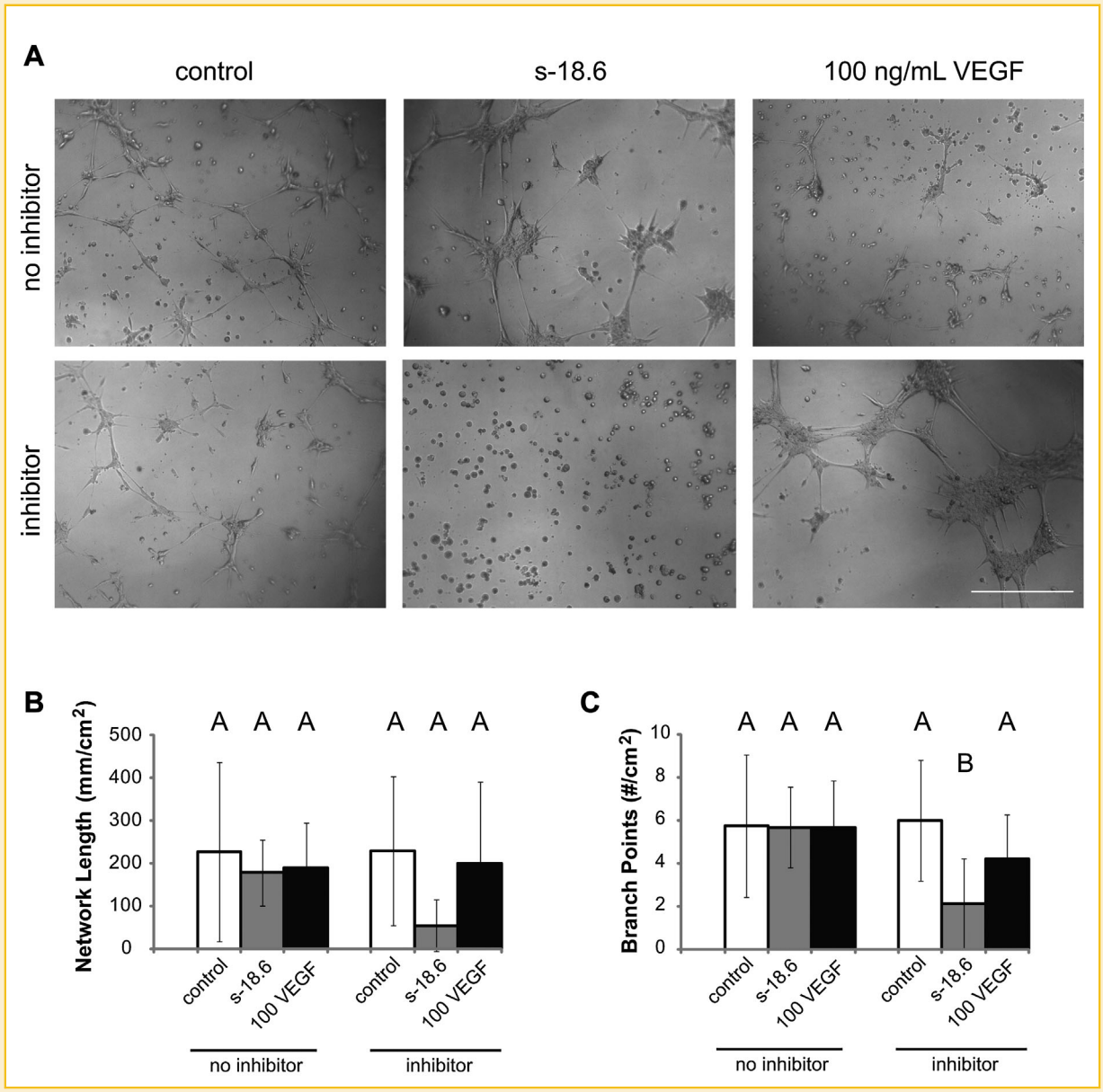

Fig. 6. VEGF surfaces acted in synergy with an inhibitor of VEGF receptors to suppress an angiogenic response. MSCs were differentiated towards endothelial cells on VEGF or TCPS surfaces with (100 VEGF) or without (control) 100 ng/ml soluble VEGF. After 7 days of differentiation, cells were seeded on ECMatrix for 5 h. A: Representative images of the angiogenic response. Scale bar represents 500 μm. B: Network length and (C) number of branch points were quantified (four images per well, n = 3). Letters indicate statistically similar groups as determined from Tukey's post hoc analysis ($P > 0.05$) of a one-way ANOVA. Error bars indicate 1 SD.

VEGF signaling. New inhibition methods are of clinical interest as elevated levels of soluble VEGF are found in environments with pathological angiogenesis, such as retinopathies [Siemerink et al., 2013] and tumors [Moserle and Casanovas, 2013]. Current VEGF inhibition methods are temporary because tumors develop drug resistance. Consequently, there has been interest in discovering new classes of inhibitors that can sustain stable vasculature around a tumor since stable vasculature is believed to be essential in preventing metastasis and increasing overall survival rates of patients [Moserle and Casanovas, 2013].

Cells seeded on s-5.9 had a decrease in proliferation when cultured in medium containing an inhibitor for VEGF receptors compared to control medium. The observed decrease in proliferation for cells on VEGF surfaces in the presence of a pharmacological inhibitor agrees with observations by Koepsel et al. [2012] but disagrees with observations by Backer et al. [2006], who observed an increase in proliferation on VEGF surfaces even in the presence of an inhibitor. Since different inhibitors were used in all three studies, it is impossible to conclude if the observed effects mainly result from the individual surfaces or the distinct inhibitors.

Suppressed proliferation was not the only effect observed on cells cultured with the inhibitor. The ability of endothelial cells derived from MSCs to form networks on ECMatrix was significantly reduced by the combination of high VEGF surface density and AZD2171 in the medium. While consistent with the observations regarding cell proliferation, this effect is somewhat surprising as bound VEGF has been speculated to increase vascular branching [Ruhrberg et al., 2002]. Furthermore, previous reports using bound VEGF in three-dimensional matrices observed increased morphogenesis and branching [Koch et al., 2006; Leslie-Barbick et al., 2009; Wang et al., 2008] as well as increases in genes associated with angiogenesis [Porter et al., 2011]. However, one significant difference is that the differentiated MSCs in our study were not in contact with surface-bound VEGF or the inhibitor once they were seeded on ECMatrix. Thus, the observed decrease in tubule formation and network branching could be due to long-term effects of contact with bound VEGF. Taken together, all of our results indicate that covalently bound VEGF can inhibit angiogenic responses to soluble VEGF or growth factors in ECMatrix.

In conclusion, our results suggest that the surface density of VEGF can be used to inhibit the angiogenic response of cells. At low surface densities (0.04–2.8 ng/cm^2), the effect of bound VEGF may be negligible, whereas at intermediate surface densities (5.8–18.6 ng/cm^2), bound VEGF may inhibit an angiogenic response. Thus, tailoring the surface density of bound VEGF may provide a new way to promote or inhibit vasculogenesis and angiogenesis.

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