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Regulating VEGF signaling in platelet concentrates *via* specific VEGF sequestering†

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Platelets contain an abundance of growth factors that mimic the composition of the wound healing milieu, and platelet-derived VEGF in particular can negatively influence wound healing if unregulated. Here, we sought to capture and regulate the activity of VEGF factor from human platelets using poly(ethylene glycol) microspheres. In this communication, we demonstrate that platelet freeze/thaw produced significantly higher levels of Vascular Endothelial Growth Factor (VEGF) than either calcium chloride treatment, protease activated receptor 1 activating peptide (PAR1AP) treatment, or thrombin treatment. PEG microspheres containing a VEGF-binding peptide (VBP), derived from VEGFR2, sequestered VEGF from platelet concentrate, prepared *via* freeze/thaw, and reduced the bioactivity of platelet concentrate in HUVEC culture, which suggests that VBP microspheres sequestered and reduced the activity of VEGF from patient-derived platelets. Here, we demonstrate the ability of VEGF sequestering microspheres to regulate the activity of VEGF derived from a growth factor-rich autologous human blood product.

Introduction

Angiogenesis during wound healing involves a complex interplay of vascular and stromal cells, the extracellular matrix, and platelets that are activated upon wound healing stimuli.[1,2] Platelet activation initiates growth factor release and subsequent growth factor signaling to cells in the wound healing milieu.[1] For example, activated platelets release Vascular Endothelial Growth Factor (VEGF), which signals to vascular endothelial cells (ECs) and initiates angiogenic sprouting during early wound healing.[3–5] Activated platelets also release other key growth factors, which promote vascular stabilization by ECs/pericytes[6] (Platelet-Derived Growth Factor BB, PDGF-BB) and stimulate fibroblast proliferation[7,8] and extracellular matrix deposition[5] (Transforming Growth Factor β1, TGFβ1). Platelets have been used clinically in the form of platelet rich plasma (PRP)[9] and other blood products. However, blood products and the wound healing milieu are heterogeneous and necessitate strategies to specifically regulate the activity of particular autologous biomolecules.

Wound healing requires a fine-tuned balance between pro- and anti-angiogenic growth factors and cytokines. Activated platelets release multiple pro-angiogenic growth factors simultaneously, and each of these factors can have unintended side effects if they are not provided within a narrow therapeutic concentration range. One illustrative example is VEGF, which is released by activated platelets and can negatively impact musculoskeletal wound healing[10,11] if unregulated. Here we describe a novel approach to specifically regulate the activity of VEGF released from activated platelets. Platelet releasate provides a valuable tool to examine the influence of growth factor regulation in an *ex vivo* context relevant to regenerative medicine. We activated platelets using a scalable freeze/thaw technique and captured and regulated the activity of VEGF from platelet concentrate using biomaterials that mimic the VEGF-binding ability of VEGF receptor type 2 (VEGFR2).

Materials and methods

Peptide synthesis

Protease activated receptor-1 activating peptide (PAR1AP), a VEGF-binding peptide (VBP) derived from VEGF receptor 2,[12] a divalent VBP (VBP_2) with the sequence KE{F_d}{A_d}{Y_d}{L_d}IDFNWEYPASKCKSAPYEWNFDI{L_d}{Y_d}{A_d}{F_d}EK, and a scrambled sequence of divalent VBP (Scr_2) with the sequence KD{A_d}PYN{F_d}EFAWE{Y_d}IS{L_d}KCK{L_d}SI{Y_d}EWAFE{F_d}NYP{A_d}DK were synthesized using fmoc solid phase peptide synthesis using a microwave peptide synthesizer (Discover; CEM) and automated liquid handling (Liberty1; CEM). All amino acids and Rink Amide MBHA resin were purchased from EMD Novabiochem. Briefly, resin was swelled in *N*,*N*-dimethyl-

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formamide (DMF; Fisher) and deprotected in 20 vol% piperidine (Sigma) and 0.1 M HOBt (Advanced Chem Tech) in DMF for 5 minutes under microwave at 70 °C. Resin was washed twice in DMF before coupling, and the de-protected resin was mixed with four molar equivalents of amino acid (dissolved at 0.2 M in DMF) relative to free amines, four molar equivalents of HBTU (Advanced Chem Tech), and eight molar equivalents of diisopropylethylamine (DIPEA; Fisher), which was dissolved at 35 vol% in *N*-methyl-2-pyrrolidone (Fisher). Coupling was performed for 5 minutes at 70 °C (cysteine was coupled at 50 °C). Peptide purity was verified using reverse phase high performance liquid chromatography. Peptide identity was verified using time-of-flight mass spectroscopy (Bruker), and peptide content was determined using an Ellman's assay (Thermo Scientific) to measure free thiols.

Synthesis of PEG-norbornene microspheres

Four-arm poly(ethylene glycol) (PEG, M_n 20 000; Jenkem) terminated on each arm with hydroxyl groups was functionalized with norbornene using carbodiimide chemistry[13] as previously described.[14] Microspheres containing no peptide (Blank), VBP, or Scramble were synthesized as previously described[13] using an aqueous emulsion of a PEG-rich phase with 10 wt% PEG-norbornene, half molar equivalent PEG dithiol (M_n = 3400; Laysan Bio) with respect to norbornene groups, photoinitiator (0.05% Irgacure 2959; Ciba), and either deionized (DI) water or dissolved peptide (VBP, Scramble) at 0.016 molar equivalents with respect to norbornene, with a Dextran-rich phase containing 40 wt% Dextran (M_n = 40 000; Alfa Aesar) in KCl buffer. For microsphere synthesis with divalent peptides, peptides were first dissolved in DI water with added ammonium hydroxide (to facilitate dissolution) and were incorporated into microspheres at a ratio of 0 (Blank), 0.002 (0.2%), 0.008 (0.8%), and 0.063 (6.3%) molar equivalents with respect to norbornene. For all microsphere types, the PEG-rich phase was emulsified *via* vortexing for one minute in a six-fold volumetric excess of the Dextran-rich phase and was exposed to UV light (1.1 J cm^{-2}). Microspheres were washed in DI water and centrifuged at 1600*g*, and the final product was then suspended in DI water, frozen in liquid nitrogen, and lyophilized. Peptide-containing microspheres were suspended in phosphate buffered saline (PBS; Fisher Scientific), and peptide content was verified using either Micro-BCA assay (Thermo Scientific) or by UV-Vis absorbance at 260 nm corresponding to the absorbance of tryptophan and tyrosine.

Platelet activation

Expired platelet packs were donated from the University of Wisconsin Blood Bank, aliquoted, and stored at 4 °C for processing on the day of receipt. Platelet aliquots were centrifuged at 2000*g* for 12 minutes, and the supernatant was collected and saved (referred to as "Plasma," P) or was used to re-suspend un-activated platelets (referred to as "Platelets + Plasma,", P + P). Platelets in treatment groups were suspended in HEPES buffer (0.2 M; pH 7.4; Fisher) at the same volume of Plasma that was removed. Platelets were then subjected to treatment with PARIAP (0.1 M, 0.01 M), Thrombin (0.4, 4 U mL^{-1}; Sigma Aldrich), $CaCl_2$ (0.5 wt%; Fisher), or HEPES buffer (control) for 30 minutes at room temperature, which is consistent with previous literature.[15–17] Platelet suspensions in freeze/thaw treatment group were subjected to freezing three times in liquid nitrogen (5 minutes) and thawing in a 37 °C water bath (10 minutes). After activation, platelet suspensions were centrifuged at 2000*g* for 10 minutes, and the supernatant (hereafter referred to as "platelet concentrate," PC) was sterile filtered through a 0.2 μm filter and collected for processing. Supernatants and controls were stored at −80 °C before ELISA was performed as described below.

Measurement of growth factors in platelet concentrate

Platelet Concentrate (PC) and both plasma (P) and platelets + plasma (P + P) controls were assayed for growth factor content using enzyme-linked immunosorbent assays (ELISA) for human Vascular Endothelial Growth Factor-165 (VEGF; R&D Systems DY293B), human Platelet-Derived Growth Factor-BB (PDGF-BB; R&D Systems DY220), or human Transforming Growth Factor β1 (TGFβ1; R&D Systems DY240) using standard assay procedure. The concentration of growth factor in each condition was calculated by comparing the corrected absorbance of each sample (450 nm–540 nm) to the standard curve generated in PBS. Samples and standard curves were assayed in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test (α = 0.05).

Incubation of microspheres with platelet concentrate

Platelets were activated using a freeze/thaw method as described above. Platelets for growth factor capture were suspended in PBS at one third of the volume of plasma removed to maximize the growth factor concentration in PC. After three freeze/thaw cycles, platelets were centrifuged as above, and the supernatant (PC) was sterile filtered and used to suspend microspheres at 1 mg mL^{-1} for 4 hours at 37 °C. After incubation, microspheres were centrifuged at 10 600*g* for 5 minutes, and the PC was subjected to the analysis methods below. For experiments with microspheres containing divalent peptides, VBP_2 or $Scramble_2$, platelets with plasma were activated by three freeze/thaw cycles and incubated directly with microspheres for 4 h at 37 °C. Subsequently, divalent peptide-containing microspheres and platelets were filtered before the filtrate was assessed using VEGF ELISA as described below.

ELISA measurement of growth factors sequestered from platelet concentrate

Microsphere supernatants as prepared above were subjected to VEGF ELISA. The concentration of VEGF in the supernatant of each condition was correlated to the amount of bound VEGF by subtracting the concentration of VEGF in the supernatant of the no microsphere condition (NS) with the concentration of VEGF in the supernatant of each respective microsphere condition. Alternatively, the supernatants generated above were stored at −80 °C before being subjected to Human Angio-

genesis/Growth Factor Magnetic Bead Panel (HAGP1MAG-12K; Millipore) as briefly described here following standard protocol. Briefly, the 96 well plate provided with the kit was washed in wash buffer, and subsequently samples (either un-diluted or diluted threefold in PBS) or standards (serially diluted in PBS) were incubated with antibody-conjugated beads and assay buffer overnight at 4 °C on a plate shaker. Beads were then washed in wash buffer, incubated with detection antibody cocktail for 1 hour at room temperature on a plate shaker, and incubated with streptavidin/phycoerythrin for 30 minutes at room temperature on a plate shaker. Finally, beads were washed with wash buffer and suspended in Magpix drive fluid (Life Technologies) for analysis on the Magpix Luminex XMAP (Life Technologies). Standard curves were analyzed using 4-PL analysis in GraphPad Prism, and median sample counts were correlated to standard curve using 4-PL interpolation to calculate a mean growth factor concentration for each dilution and microsphere condition. Data was aggregated from two separate readings of triplicate samples that were prepared at two different dilutions. Statistical analysis of the multiplexed ELISA was performed using one-way ANOVA with Fisher's least significant difference *post-hoc* test (α = 0.01), and plate-based ELISA data was analyzed using one-way ANOVA with Bonferroni *post-hoc* test (α = 0.05).

Regulation of the activity of VEGF captured from PC

Human umbilical vein endothelial cells (Lonza) were expanded under normal culture conditions (37 °C, 5% CO_2) in medium 199 (M199; CellGro) supplemented with EGM-2 BulletKit (Lonza) and penicillin/streptomycin (P/S; Gibco) and were used at passage 4 for experiments. On the day before experiments, HUVECs were seeded overnight under normal culture conditions onto 96 well plates, which were pre-coated with fibronectin (Corning), at 4000 cells per well in M199 supplemented with 2 vol% fetal bovine serum (FBS; Gibco) and P/S. On the day of experiments, microspheres (VBP, Scramble, and Blank) were sanitized with 70 vol% ethanol (Fisher) in DI water for 1 hour and washed with PBS prior to use. PC was generated as described above using freeze/thaw, and microspheres were subsequently incubated in either 0.1% BSA in PBS or PC at 1 mg mL^{-1} microspheres for 3 hours at 37 °C. Microspheres were centrifuged at 1600*g* for 5 minutes and washed for 1 hour in 0.1% BSA in PBS at 37 °C. Microspheres were again centrifuged at 1600*g* for 5 minutes and subsequently suspended at 5 mg mL^{-1} in M199 supplemented with 2 vol% FBS and P/S. Medium from HUVEC culture was replaced with microsphere suspensions, and HUVECs were cultured with microspheres for 48 hours under normal culture conditions in the presence of 10 μM EdU (Thermo Scientific). After 48 hours, HUVECs were fixed in formalin (Fisher), stained with AlexaFluor 594, and counter-stained with Hoechst using standard Click-iT EdU (Thermo Scientific) assay protocol as described previously.[13] The mean fraction of EdU+/Hoechst+ cells was tabulated for 6 replicate well per condition, and statistical analysis was performed using one-way ANOVA and Dunnet's *post-hoc* test or two-way ANOVA (α = 0.05).

Results and discussion

The relative growth factor concentrations in platelet concentrate (PC) varied depending on the method used for platelet activation: thrombin treatment,[15,17,18] PAR1AP treatment,[18,19] $CaCl_2$ treatment,[17] or freeze/thaw.[20,21] In particular, each activation method elicited different concentrations of three model growth factors that are critical during wound healing[5] – VEGF-A, PDGF-BB, and TGFβ1. Here, we specifically characterized the abundance of a particular VEGF-A isoform, $VEGF_{165}$, in platelet concentrate, as megakaryocytes primarily secrete $VEGF_{165}$ upon activation by thrombin.[15] Freeze/thaw resulted in a substantially higher concentration of released $VEGF_{165}$ (hereafter referred to as 'VEGF') relative to all activation conditions (Fig. 1A) and a higher concentration of released TGFβ1 relative to all activation conditions except 4 U mL^{-1} Thrombin (Fig. 1C). 4 U mL^{-1} Thrombin resulted in the highest concentration of released PDGF-BB in PC relative to all other conditions (Fig. 1B). Substantially higher concentrations of each

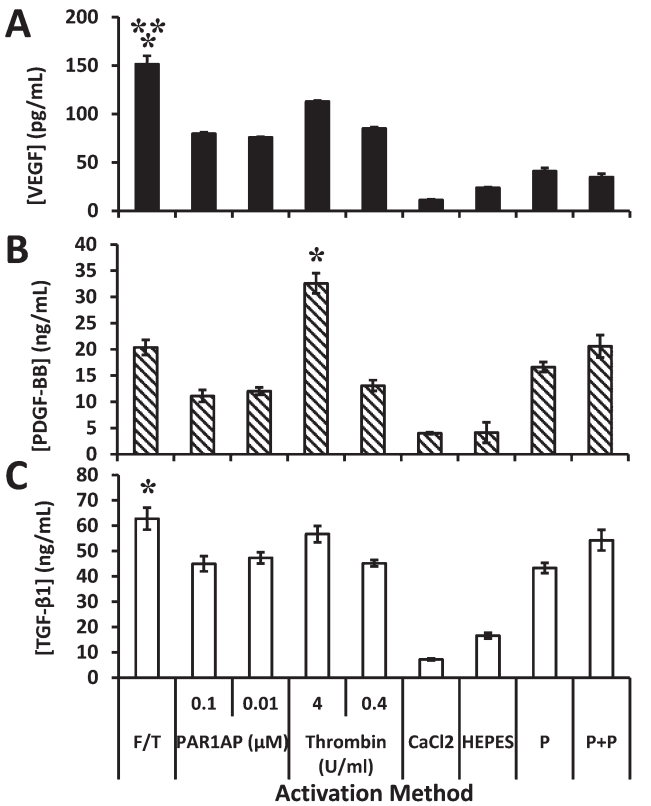

Fig. 1 Measurement of VEGF, PDGF-BB, and TGFβ1 present in platelet concentrate prepared by various methods. Quantification of VEGF165 (here referred to as VEGF) (A), PDGF-BB (B), and TGFβ1 (C) concentration in the supernatant of platelets prepared by the methods (described in Materials and Methods) listed on the *x*-axis below each bar. Statistical comparisons were made using one-way ANOVA with Bonferroni *post-hoc* test and is shown for *p*-value < 0.05 relative to 4 U mL^{-1} thrombin (**) or relative to all methods except thrombin(*). F/T = freeze/thaw, PAR1AP = protease activated receptor 1-activating peptide, P = plasma, P + P = platelets with plasma.

growth factor were present in PC generated using each technique relative to inactive platelets in the HEPES only control, as expected (Fig. 1). Differences in growth factor released by each respective technique may be due to the differential capacity of each growth factor to bind to the fibrin clot produced during platelet activation.[22] During freeze/thaw, no fibrin clot was formed, which suggests that each growth factor was not sequestered by fibrin and was thus soluble after freeze/thaw and centrifugation. The concentrations of VEGF released here *via* freeze/thaw or thrombin treatment are comparable to those reported in previous studies of thrombin-activated platelets.[17] Further, while freeze/thaw and thrombin activation techniques employed here released TGFβ1 at concentrations comparable to those previously reported in the literature,[20] the concentration of PDGF-BB released here after freeze/thaw or thrombin activation was almost one order of magnitude higher than previously reported for thrombin activation.[20] Literature supports the ability of freeze/thaw to maintain the immunoreactivity and activity of platelet-derived growth factors,[20,23,24] and based on our observation that freeze/thaw produced the highest concentration of immunoreactive VEGF relative to the other activation conditions (Fig. 1A), we used freeze/thaw to generate PC for subsequent analysis of growth factor sequestering (Fig. 2A) and regulation.

Microspheres containing VEGF-binding peptide motifs (VBP microspheres) sequestered primarily VEGF from PC. To evaluate the specificity of VEGF sequestering, we performed a bead-based multiplexed ELISA to characterize sequestering of 12 model proteins associated with angiogenesis. Analysis of multiplexed ELISA data demonstrated that VBP microspheres sequestered VEGF from PC generated *via* freeze/thaw (Fig. 2B). Angiopoietin-2, Placental Growth Factor (PlGF), and Vascular Endothelial Growth Factor-D (VEGF-D) were not present in PC at detectable levels. While Interleukin-8 (IL-8), Heparin-Binding Epidermal Growth Factor-like Growth Factor (HB-EGF), Fibroblast Growth Factor-1 (FGF-1), and VEGF-C were present in PC at low concentrations in PC (<100 pg ml^{-1}), none of these growth factors showed detectable sequestering to any of the microsphere conditions evaluated (Fig. 2B). The PC incubated with or without microspheres also contained moderate levels of FGF-2 (~120 pg mL^{-1}), VEGF-C (~150 pg mL^{-1}), Epidermal Growth Factor (EGF) (~500 pg mL^{-1}), and Leptin (~1500 pg mL^{-1}), and none of these growth factors showed detectable sequestering to any of the microsphere conditions evaluated (Fig. 2B). The PC incubated with VBP microspheres and microspheres containing a scrambled version of VBP (Scramble) contained a significantly lower concentration of VEGF when compared to NS control, and the level of sequestered VEGF (~200 pg per mg microspheres) was on the order of the ED_{50} of VEGF (100–200 pg mL^{-1}).[25] We observed no statistical differences in VEGF content in the supernatant of VBP microspheres compared to either Scramble microspheres or microspheres without peptide (Blank) in the multiplexed ELISA (Fig. 2B), and therefore we sought to further interrogate the extent of VEGF sequestering to VBP microspheres using a plate-based ELISA.

VBP microspheres sequestered significantly more VEGF from PC than Scramble or Blank microspheres. Specifically, VBP microspheres sequestered ~20% of the VEGF present in PC (relative to the NS control) (Fig. 2C). Neither Blank nor Scramble microspheres sequestered a significant amount of VEGF relative to the no microsphere (NS) control (Fig. 2C). The extent of VEGF sequestering to VBP microspheres here (~120 ng out of ~600 ng total VEGF in PC, or ~20% VEGF sequestering) is generally consistent with the ability of VBP microspheres to sequester ~40% of the recombinant VEGF in albumin-containing medium or 10–60% of the recombinant VEGF in serum-containing medium as previously demonstrated.[13,26] Here we provide the first demonstration of VEGF sequestering from a patient-derived autologous blood product that is relevant to the VEGF content of the wound healing milieu.[27] Taken together, these results demonstrate that VBP microspheres sequestered VEGF with specificity, and we further hypothesized that VBP microspheres would influence VEGF activity in PC without influencing the activity of other pro-angiogenic platelet-derived GFs.

VBP microspheres reduced the influence of PC on HUVEC proliferation, indicating that VEGF sequestering from PC was biologically significant. Culture with microspheres pre-incubated in PC resulted in an increased fraction of HUVECs in S-phase when compared to culture with microspheres pre-incubated in PBS and to the no VEGF control (Fig. 3A). The stimulation of HUVEC proliferation in response to microspheres pre-soaked in PC corroborates a similar finding that gelatin microspheres manufactured in platelet rich plasma stimulated endothelial cell pro-angiogenic function *in vitro* and increased ischemic limb reperfusion *in vivo* relative to microspheres manufactured in platelet poor plasma.[28] Interestingly, VBP microspheres pre-incubated in PC reduced HUVEC proliferation relative to both Scramble and Blank microspheres pre-incubated in PC (Fig. 3A). This result agrees with a previous study, which demonstrated that VBP microspheres reduced HUVEC proliferation in culture with recombinant VEGF.[13] Together with our observation that VBP microspheres specifically sequestered VEGF from PC (Fig. 2), we conclude that VBP microspheres reduced VEGF-dependent HUVEC proliferation by decreasing the concentration of free soluble VEGF relative to control microspheres. This conclusion is substantiated by previous evidence that VBP microspheres sequestered and reduced VEGF activity in culture[13] and retained VEGF over a much longer time-frame than either Scramble or Blank microspheres.[29]

We hypothesized that microspheres increased HUVEC proliferation after incubation with PC as a result of mass transport of PC-derived proteins into hydrogel microspheres[30] (Fig. 3B). We confirmed the presence of several unique proteins (including a protein band consistent with VEGF at approximately 37 kDa) that were present in all microsphere types after incubation with PC (Fig. 1SA†), which suggests that several PC-derived proteins were present in microspheres after incubation with PC, released into HUVEC culture, and stimulated HUVEC proliferation. However, densitometry analysis of the normal-

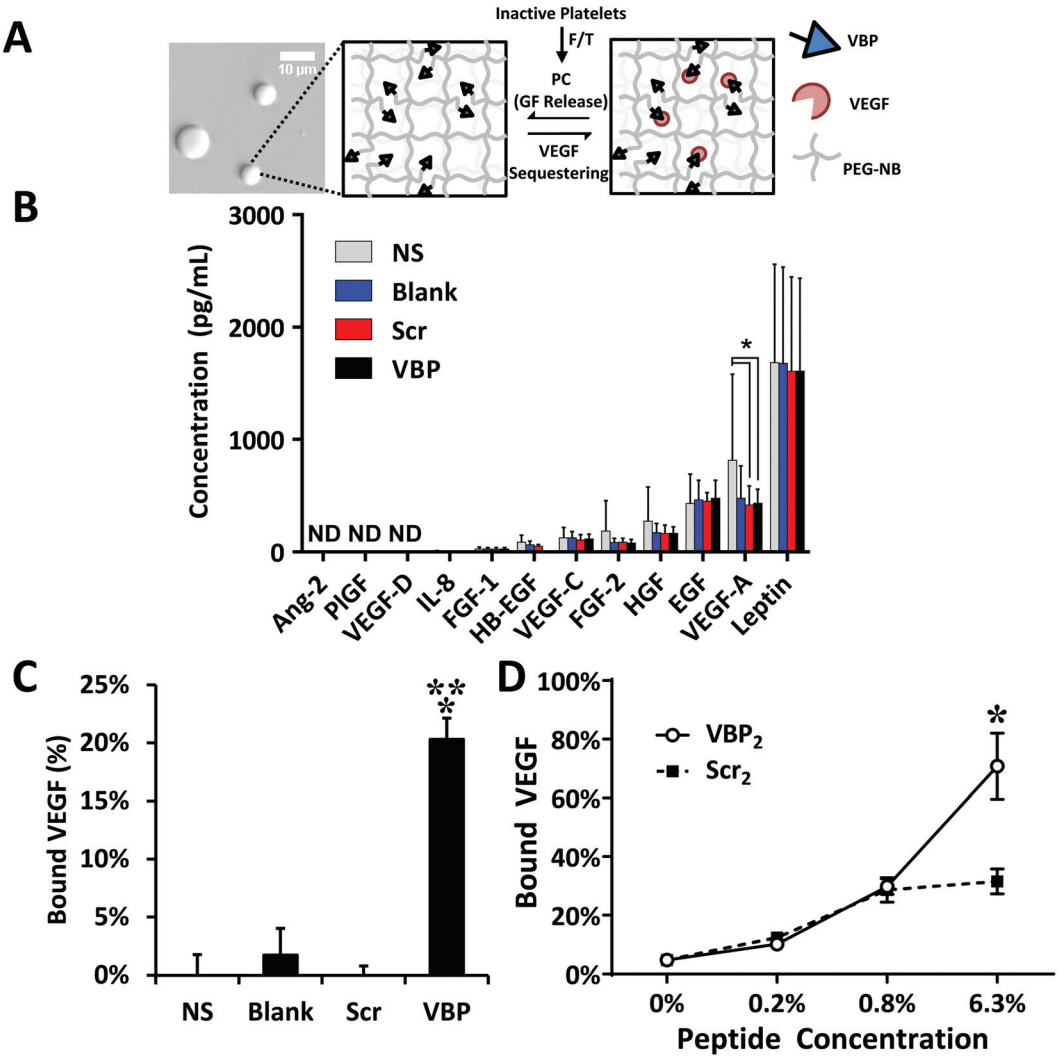

Fig. 2 VBP Microspheres Sequester VEGF from PC Prepared *via* Freeze/Thaw. A: Schematic of platelet activation and subsequent VEGF sequestering to VBP microspheres incubated in PC. B: Multiplexed bead-based ELISA was performed to assess the abundance of 12 pro- angiogenic growth factors in the supernatant after incubation of microspheres with PC. Data was compared to standard curve generated using recombinant growth factors in PBS, and the abundance of each growth factor in supernatant was calculated from the standard curve using 4- PL analysis in GraphPad Prism. ND = Not Detected. Statistical comparisons were made using one-way ANOVA with Fisher's least significant difference *post-hoc* test. Statistical significance is denoted for p-value < 0.05 (*) between the conditions indicated in brackets. C: Quantification of VEGF sequestering to VBP microspheres (in % bound VEGF) calculated by subtracting the concentration of VEGF in the no microsphere control (NS) by the concentration of VEGF in each respective microsphere condition and dividing by the total amount of VEGF in the NS control. Statistical comparisons were made using one-way ANOVA and Bonferroni *post-hoc* test and is denoted for p-value < 0.05 relative to Scramble (**) and Blank (*) microspheres. D: VEGF sequestering to dimeric VBP, VBP_2, and dimeric Scramble, Scr_2, at varying peptide concentration (presented as % peptide per norbornene group during microsphere synthesis with PEG-NB). % Bound VEGF was calculated as described above. Statistical analysis was performed using two-way ANOVA (peptide identity, peptide concentration, and interaction p-value < 0.05) with *post-hoc* Bonferroni test denoted for p-value < 0.05 with an asterisk (*) comparing VBP_2 and Scr_2 at each peptide concentration.

ized intensity of the 37 kDa protein band did not result in statistical differences between VBP, Scramble, and Blank microspheres (Fig. 1SB†). Our data together suggest a mechanism whereby PC-derived proteins (including VEGF) diffused into all microsphere types (Fig. 3B) and stimulated HUVEC proliferation upon release in culture, while VBP microspheres sequestered significantly more VEGF than controls (Fig. 2) and reduced the amount of soluble VEGF in culture.

Finally, previous results have demonstrated that microspheres containing divalent VBP sequestered VEGF to a greater extent and with higher affinity than microspheres containing monomeric VBP,[31] and thus we hypothesized that this approach could be used to increase the efficiency of VEGF sequestering from PC. We activated platelets (with plasma) *via* freeze/thaw and demonstrated that VEGF sequestering to microspheres containing a high concentration of divalent VBP,

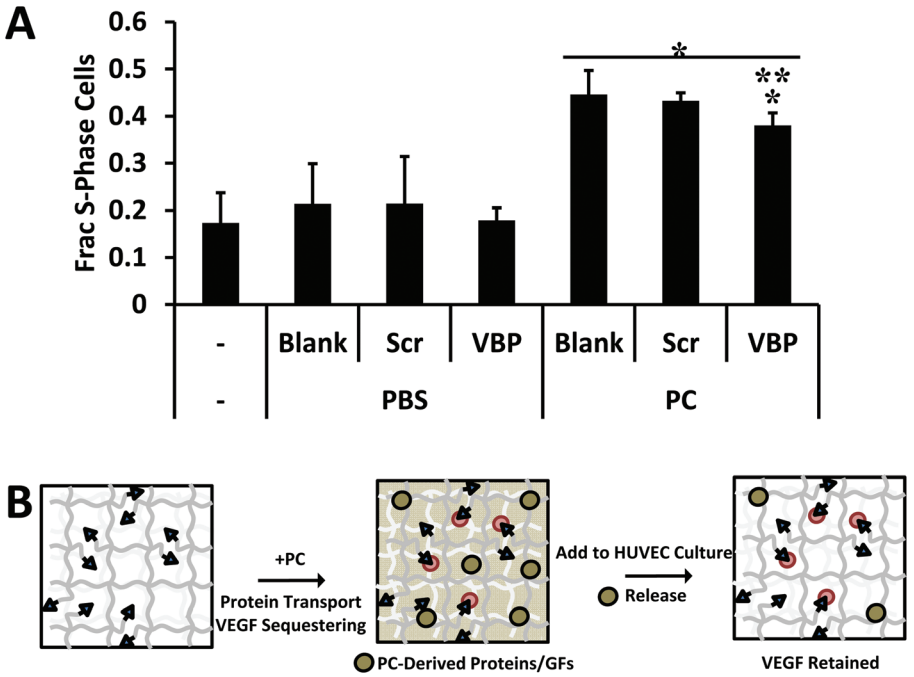

Fig. 3 VBP Microspheres Reduce HUVEC Proliferation After incubating in PC. A: Quantification of EdU+/Hoechst+ HUVEC nuclei, denoted as the fraction of HUVECs in S-phase, in the no VEGF control (–) and in the presence of microspheres either pre-incubated in PBS or pre-incubated in PC. Statistical analysis was performed relative to Blank and Scramble control microspheres in either PBS or PC. Statistical significance among each group (PBS or PC) was performed using one-way ANOVA with *post-hoc* Dunnett test and is denoted for p-value < 0.05 compared to Scramble (**) and Blank (*) microspheres. Alternatively, statistical analysis was performed relative to each respective microsphere condition incubated in PC or PBS using two-way ANOVA, and statistical significance is denoted for p-value < 0.05 (*). B: Schematic demonstrating the hypothesized mechanism whereby microspheres incubated in PC took up PC-derived proteins and GFs *via* mass transport. We hypothesize that VBP microspheres specifically sequestered and retained PC-derived VEGF in culture with HUVECs and released other pro-angiogenic GFs and proteins in culture. Since Scramble and Blank did not exhibit VEGF sequestering in our previous experiments, any PC-derived proteins or GFs taken up during PC incubation (including VEGF) would have been passively released *via* mass transport into HUVEC culture.

VBP_2, was significantly higher (~80%) than sequestering to Scr_2 microspheres (~30%) or Blank microspheres (~5%) (Fig. 2D). This result suggests that microspheres containing divalent growth factor-binding peptides may efficiently bind and potently reduce the activity of particular endogenous growth factors.

Here we show for the first time that VEGF-sequestering microspheres sequestered and regulated the activity of VEGF derived from an autologous blood product. This approach may be important, as the presence of high concentrations of VEGF in the wound healing milieu can negatively impact musculoskeletal wound healing.[10,11] Further, previous efforts have found difficulty standardizing blood-derived therapeutics and establishing their clinical safety and efficacy.[9] Therefore, the concept of regulating specific growth factors known to be present at therapeutic levels in blood products may result in a more precise control over therapeutic effects. While we focused this communication on VEGF sequestering, a similar biomaterials approach could be used to sequester other growth factors abundant in blood products, such as TGFβ1 and PDGF-BB, which can both elicit pleiotropic effects on wound healing.[5,32,33] Future studies may approach an ultimate goal of precisely controlling the abundance of endogenous growth factors in the wound healing milieu.

Conclusion

Platelets are a clinically relevant source of growth factors for wound healing applications, but the abundance of pro- and anti-angiogenic growth factors and cytokines in the wound healing milieu necessitates strategies to regulate the activity of particular growth factors that may exhibit detrimental effects on wound healing. Here, we determined that a platelet freeze/thaw cycle produced higher levels of VEGF than treatment with thrombin, PAR1AP, or calcium chloride. Given the abundance of VEGF in platelets and the need to tightly regulate VEGF activity during wound healing, we sought to specifically sequester and regulate platelet-derived VEGF using poly(ethylene glycol) microspheres containing a VEGF-binding peptide (VBP) derived from VEGFR2. Out of a panel of 9 measured growth factors in platelet concentrate, VBP microspheres sequestered primarily VEGF, and VBP microspheres pre-incubated in platelet concentrate reduced HUVEC proliferation relative to controls. We posit that a similar biomimetic approach could be broadly applied to regulate the activity of other pro-angiogenic growth factors derived from autologous blood products (*e.g.* TGFβ1, PDGF-BB) using biomimetic biomaterials. In summary, we demonstrate here that VBP microspheres specifically sequestered and regulated the activity of

VEGF from an endogenous human source, which highlights a therapeutic application of VBP microspheres to reduce VEGF activity in regenerative applications.

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