Cell-Demanded Liberation of VEGF\textsubscript{121} From Fibrin Implants Induces Local and Controlled Blood Vessel Growth

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Abstract—Although vascular endothelial growth factor (VEGF) has been described as a potent angiogenic stimulus, its application in therapy remains difficult: blood vessels formed by exposure to VEGF tend to be malformed and leaky. In nature, the principal form of VEGF possesses a binding site for ECM components that maintain it in the immobilized state until released by local cellular enzymatic activity. In this study, we present an engineered variant form of VEGF, \(\alpha_{\text{r}}\text{PI}_{1-8}\)-VEGF\textsubscript{121}, that mimics this concept of matrix-binding and cell-mediated release by local cell–associated enzymatic activity, working in the surgically-relevant biological matrix fibrin. We show that matrix-conjugated \(\alpha_{\text{r}}\text{PI}_{1-8}\)-VEGF\textsubscript{121} is protected from clearance, contrary to native VEGF\textsubscript{121} mixed into fibrin, which was completely released as a passive diffusive burst. Grafting studies on the embryonic chicken chorioallantoic membrane (CAM) and in adult mice were performed to assess and compare the quantity and quality of neovascularure induced in response to fibrin implants formulated with matrix-bound \(\alpha_{\text{r}}\text{PI}_{1-8}\)-VEGF\textsubscript{121} or native diffusible VEGF\textsubscript{121}. Our CAM measurements demonstrated that cell-demanded release of \(\alpha_{\text{r}}\text{PI}_{1-8}\)-VEGF\textsubscript{121} increases the formation of new arterial and venous branches, whereas exposure to passively released wild-type VEGF\textsubscript{121} primarily induced chaotic changes within the capillaryplexus. Specifically, our analyses at several levels, from endothelial cell morphology and endothelial interactions with periendothelial cells, to vessel branching and network organization, revealed that \(\alpha_{\text{r}}\text{PI}_{1-8}\)-VEGF\textsubscript{121} induces vessel formation more potently than native VEGF\textsubscript{121} and that those vessels possess more normal morphologies at the light microscopic and ultrastructural levels. Permeability studies in mice validated that vessels induced by \(\alpha_{\text{r}}\text{PI}_{1-8}\)-VEGF\textsubscript{121} do not leak. In conclusion, cell-demanded release of engineered VEGF\textsubscript{121} from fibrin implants may present a therapeutically safe and practical modality to induce local angiogenesis. (Circ Res. 2004;94:1124-1132.)

Key Words: therapeutic angiogenesis ■ vascular endothelial growth factor ■ fibrin ■ controlled release

In many diseases of ischemia, eg, peripheral vascular disease, coronary ischemia, and chronic wounds, the intrinsic capacity for spontaneous vascular repair and tissue regeneration is severely compromised. Treatment of these pathologies by therapeutic angiogenesis, ie, biochemical stimulation of collateral vessel formation, has been proposed by administering angiogenic growth factors such as vascular endothelial growth factor (VEGF) or one of the fibroblast growth factors (FGFs) as recombinant proteins, genes, or factor-overexpressing cell transplants. Indeed, preclinical and initial clinical trials have shown that delivery of VEGF or FGF can improve regional blood flow in underperfused heart or legs.\(^1\) These early studies have provided encouragement; however, pharmacological issues seem to limit therapeutic angiogenesis: bolus injections or systemic delivery of VEGF or bFGF proteins showed low efficacy due to rapid clearance from the target site, and edema and hypotension can result due to the tendency of VEGF to increase vascular permeability. Furthermore, high local levels of VEGF can induce unregulated formation of supernumerary, but malformed vessels in hemangioma-like assemblies.\(^2-3\)

These pharmacokinetic and safety issues have spurred the development of biopolymeric carriers to regulate VEGF’s persistence, release rate, and availability at the target site. A variety of natural and synthetic materials have been used as carriers, including the biological hydrogel matrix fibrin, which can be surgically applied as sealant and adhesive in “fibrin glue” formulations formed from plasma cryoprecipitate.\(^4\) Fibrin naturally forms in immediate response to vessel injury and tissue damage and thus serves as a natural provisional material platform for regeneration. While new tissue is forming, fibrin is gradually degraded by plasmin or

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matrix metalloproteinases (MMPs) produced in the local milieu at the surface of cells that invade the matrix. Because fibrin lyses slowly and locally, it has been used as a reservoir for angiogenic proteins, cells, and genes. In spite of some positive results with simple admixtures of FGF or VEGF proteins in fibrin glue, the release kinetics of such preparations are indicative of an uncontrolled burst. Under these conditions, the activity of VEGF may become adverse to healing.

Our laboratory has developed an approach to prevent rapid clearance of growth factors from fibrin by covalently incorporating the factor within fibrin to couple its release to the local proteolytic activity associated with cells invading the matrix. Indeed, such coupling of growth factor release with local cellular activity is very important in nature. For example, the principal VEGF isoform found in tissue, VEGF165, associates with heparan sulfate proteoglycans in the extracellular matrix (ECM), which stabilizes its active conformation, protects it from proteolytic inactivation, and limits its availability to regions of active cell invasion. Inhibitors of MMPs, plasmin and heparinases present in biological fluids serve to localize this effect to the surfaces of cells invading the matrix, at which active enzyme is continually produced.

In this study, we used an engineered variant, α2PIe8-VEGF121, that mimics this concept of matrix-binding and regulation of release by cell-associated proteolytic activities (Figure 1A). α2PIe8-VEGF121 represents a bidomain protein construct composed of a fibrin-coupling factor XIIIa substrate site from α2-plasmin inhibitor7 and mature human VEGF121. Covalent coupling to fibrin matrix provides retention of α2PIe8-VEGF121 until its proteolytic liberation during fibrin resorption and remodeling.

Materials and Methods

Vascular Endothelial Growth Factors
The preparation of recombinant α2PIe8-VEGF121, containing the factor XIIIa substrate sequence NQQEVSPL at the amino terminus of mature human VEGF121, has been described. Similarly, native sequence human VEGF121 was cloned and expressed in the bacterial expression plasmid pRSET (Novagen) (see the expanded Materials and Methods in the online data supplement available at http://circres.ahajournals.org).

Fibrin Gel Matrices Formulated With VEGF
Fibrin gel matrices were prepared by mixing the following components at the final concentrations of 10 mg/mL fibrinogen (Fluka AG), 2 U/mL factor XIII (kindly provided by Baxter AG, Vienna), and 2.5 mmol/L CaCl2. α2PIe8-VEGF121 or VEGF121 were mixed within the fibrinogen solution before initiation of fibrin gelation by addition of thrombin.

Mitogenic Activity of Matrix-Derived VEGFs
The activities of matrix- liberated VEGF forms were determined in human umbilical vein endothelial cell (HUVEC; PromoCell) proliferation assays as described in the expanded Materials and Methods.

Determination of VEGF Release Profiles
Release of VEGF was studied by incubating 50 μL fibrin gel matrices gels formulated with 2 μg of α2PIe8-VEGF121, or native VEGF121 in 10 mL washing buffer (TBS, 0.1% BSA) for 24 hour at 37°C with occasional shaking. Aliquots (100 μL) of the incubation buffers were taken after 1, 2, 4, 8, and 24 hours. VEGF amounts in the aliquots were assessed using the Duoset Human VEGF ELISA kit (R&D Systems). Percentages of released VEGFs were calculated using the ELISA values obtained for direct dilutions of 2 μg VEGF in washing buffer as a reference for total release.

Chick Chorioallantoic Membrane Assay and In Vivo Microscopy
Chicken embryos were cultured by the shell-free method. Disc-shaped 40 μL fibrin gel matrices were grafted atop the growing chicken chorioallantoic membrane (CAM) at embryonic day 9 and cultured for 2 days. In vivo microscopy was performed as described previously.

Methylmethacrylate Mercox Corrosion Casting
Mercox corrosion casts of CAM microvasculature were prepared as described.
Histological Processing and Electron Microscopy
Semithin (1 µm) sections and ultrathin sections (80 to 90 nm) of fixed and embedded CAM specimens were prepared as described and viewed with an Olympus AH-2 light microscope or a Philips EM 400 electron microscope.

Morphometric Analysis of Embryonic Chicken CAM Vasculature
Gray scale prints from two still video images per zone were used for assessment of mean vascular length density, and area density of “brush-like” vascular regions. The vasculature was skeletonized on gray scale prints and analyzed by the method of grid intersection (see expanded Materials and Methods).

Mouse Subcutaneous Teflon Chamber Implant Model
A new angiogenesis assay in mice was established to assess vessel leakage and vessel density in tissue forming in contact with gel matrix implants. The assay protocol is detailed in the expanded Materials and Methods.

Quantification of Tie2, VEGF-R2/Fk1, and VEGF-R1/Flt1
Tissue levels of VEGF-R2/Fk1 or VEGF-R1/Flt1 were determined using commercial ELISA kits (QuantiKine kit; R&D Systems). ELISA for Tie2 was performed using as capture antibody the anti–Tie-2 antibody clone33 (Upstate; LucernaAG), and as the detection antibody the goat polyclonal anti-Tie2 antibody (R&D Systems, catalogue No. AF762). Details of the protocol are given in the expanded Materials and Methods.

Statistics
Mean values and standard deviation (SD) are reported. The morphometric and ELISA data were comparatively analyzed using a two-tailed Student t test. Significance level was set at a value of P<0.05.

Results
We recently introduced the methodology of factor XIIIa-mediated covalent incorporation of α,PI-s-VEGF121 into fibrin gel matrices during coagulation9 (Figure 1A). In the present study, the impact of this coupling method on the kinetics of VEGF121 release, on the potency of angiogenic induction, and on the morphology of the resulting neovasculature were characterized and compared with conventional fibrin admixtures with native, freely diffusible VEGF121.

VEGF Release Profiles From Fibrin Formulations and Activity
The α,PI-s-VEGF121 variant demonstrated profound differences in release characteristics compared with the native VEGF121 admixed within fibrin (Figure 1B). Consistent with efficient coupling to fibrin gel matrix, the levels of α,PI-s-VEGF121 released into buffer remained low, 10.6±1.6% of the initially added α,PI-s-VEGF121 being released at 24 hours. In contrast, native VEGF121 was released completely, 100.0±1.2% at 24 hours, as a passive diffusive burst.

Conjugation of α,PI-s-VEGF121 to fibrin networks did not mask its receptor-binding site, or compromise its activity. We incorporated α,PI-s-VEGF121 or VEGF121 into fibrin gel matrices, and subsequently liberated the matrix-bound α,PI-s-VEGF121 or free VEGF121 into solution by means of plasmin-mediated degradation of fibrin. The mitogenic activities of matrix-activated α,PI-s-VEGF121 or native VEGF121 in HUVEC proliferation assays were statistically indistinguishable (Figure 1C).

Hence, engineered α,PI-s-VEGF121 in fibrin functions as designed: it was protected from clearance by diffusive burst; when liberated, it retained its ability to activate endothelial cells.

CAM Vascular Responses to Cell-Demanded or Diffusive Burst VEGF Release
Growing CAMs were exposed between embryonic days 9 and 11 to fibrin gel matrix grafts formulated with (1) 2 µg native VEGF121, (2) 5 µg α,PI-s-VEGF121, or (3) no VEGF. The CAM microvasculature was imaged by perfusion with FITC-dextran combined with time-lapse video fluorescence microscopy.11 Fibrin itself was found to induce a weak vascular irritation restricted to the application site (Figures 2A and 2B). Nevertheless, the vascular bed was regularly organized in tree-like structures with normal and spatially uniform distributions of capillaries and feeding vessels, ie, arterial and venous branches. In contrast, exposure of the CAM to fibrin formulated with VEGF121 resulted in extensive new vessel formation associated with chaotic perturbations of the capillary plexus and massive disturbance of the hierarchy of the arterial/venous tree and its connectivity with the capillary bed (Figures 2C and 2D). Many of the newly formed vessel branches were characterized by malformed, corkscrew-like structures (Figure 2D, indicated by arrowheads). Furthermore, many of those branches appeared to abruptly drain into zones of irregular capillary enlargement and growth (“brush-like” zones; arrows) that were found both highly enlarged in area and in number in the graft region (see later). The associated elongation of the perfusion distance, ie, the capillary path from an artery to a vein, is likely to cause inefficient diffusion of nutrients and oxygen.

In stark contrast, fibrin formulated with α,PI-s-VEGF121 evoked a much more normal and controlled vessel growth. Although the increase in densities of arterial/venous and capillary vessels was profound, both vascular hierarchy as well as vessel morphology of microvasculature remained regular (Figures 2E and 2F). These expanded normally structured vascular beds could provide enhanced perfusion which, together with increased vascular exchange surface area, constitute a desirable effect of successful proangiogenic treatment.

Morphometric Analysis of Induced CAM Vasculature
For quantitative morphometric analysis of CAM angiogenesis, two parameters were used, namely the mean vascular length density as an indicator of microvascular growth (Figure 3, top) and the area density of brush-like regions as an indicator of perturbed, diffuse capillary growth (Figure 3, bottom). Images of FITC-labeled CAM vasculature were acquired from concentric optical zones (labeled near-to-far in alphabetical order a to g) located 0 to 11.7 mm away from the margin of the application site. Exposure of CAM tissue to plain fibrin did not alter vascular length density at any location around the implant site (Figure 3, top). Mean vascular length density was increased by α,PI-s-VEGF121 by
116±24.6% over controls in the implant-contacting zone a. This effect remained significant within four optical zones located within a 6.3-mm radius from the application site. Comparatively poor enhancement of mean vascular length density was induced by native VEGF121 released from fibrin, namely only 42.1±29% (P<0.05 relative to α3PI1–8–VEGF121). Moreover, its effect was limited to the zone immediately adjacent to the implant.

Brush-like areas as a marker of nonphysiological angiogenesis were increased 7-fold, ie, from 5±1.7% to 34.8±6.9%, induced by diffusive burst release of native VEGF121 in the zone a bordering the implant versus plain fibrin (P<0.05; Figure 3, bottom); this enhancement was statistically significant versus fibrin alone within the inner four optical zones. Substantially less brush-like capillary growth was induced by α3PI1–8–VEGF121–fibrin (Figure 3, bottom); at the implant-facing zone a, the brush-like area density was increased to 22±5% (P<0.05 versus fibrin with VEGF121).

Corrosion Cast Analysis of Developing Vasculature
Casting provided high-resolution 3-D images of the induced CAM vasculature. A modest perturbation of regular CAM structure was observed in response to fibrin alone (Figure 4A). Capillary density in the area precisely underneath the fibrin graft appeared reduced, presumably due to insufficient contact between CAM and the ambient atmosphere (Figure 4B). Grafting of fibrin formulated with native VEGF121 resulted in increased densities both in the arterial/venous vessel layer as well as the capillary plexus, the latter showing rampant, chaotic capillary invasion throughout the underlying mesenchyme (Figures 4C, 4E, and 4G). Asterisks in Figure 4C mark assemblies of abnormal and irregular-formed worm-like capillaries. Arterial/venous vessel branches were malformed, showing corkscrew-like structures with atypical branching points and massive alterations in diameter. Connectivity between the arterial/venous layer and capillary plexus appeared severely disturbed: we found saccular-like enlarged terminal arterioles and venules with abrupt changes of diameter on their transition into capillaries (Figure 4E, arrowheads). Frequently, we observed brush-like endpoint structures in which branches were split by tissue pillars, as is characteristic for intussusceptive vessel growth (Figures 4E and 4G). These abnormalities are likely to perturb normal flow dynamics and impair perfusion and tissue supply with nutrient and oxygen.

Angiogenesis induced by α3PI1–8–VEGF121 was markedly better controlled: despite the robustness of the response, the vessel branching pattern was characterized by a well-developed arterial/venous tree with regular hierarchical levels and binary or triple branching points of normal appearance.
Further, shape and pattern of arterioles and venules connected to the capillary plexus appeared normal (Figure 4H).

**Ultrastructural Analysis of VEGF-Induced Vasculature**

Vascular structure and vessel wall assembly were further histologically examined (Figure 5). Toluidine blue-stained semithin cross sections demonstrated excessive, nonphysiological angiogenic effects of VEGF$_{121}$ burst release in all layers of the CAM. A multitude of clusters of densely packed capillaries scattered throughout the mesenchyme were detected (white asterisks in Figures 5A and 5C). In contrast, under conditions of cell-demanded release of $\alpha_3$PI$_{1-8}$-VEGF$_{121}$, such capillary clustering was rare and restricted to the subepithelial region (Figures 5B and 5D).

Transmission electron microscopic (TEM) analysis of CAM microvasculature exposed to diffusive VEGF$_{121}$ revealed several features typical for an activated endothelium: the luminal surfaces appeared irregular and rough as a result of numerous endothelial cell protrusions into the lumen, loose associations between endothelial and periendothelial cells, frequent irregular or even missing basement membrane, and abluminal endothelial protrusions reminiscent of vessel sprouts (Figure 5E). Curiously, frequent accumulations of cells inside the vessel lumen were observed: it is unclear whether these result from endothelial cell proliferation inside the lumen or from circulating cells docking to VEGF-stimulated vessels. In contrast, TEM images of vessels induced by $\alpha_3$PI$_{1-8}$-VEGF$_{121}$ showed intact vessel structures:

**Figure 3.** Quantitative effects on mean vascular length density, as an indicator of formation of arterial/venous branches, and brush-like area density, as an indicator of perturbed diffuse capillary growth, in developing chicken CAM tissue exposed to fibrin implants formulated with no VEGF, or freely diffusible native VEGF$_{121}$, or matrix-bound $\alpha_3$PI$_{1-8}$-VEGF$_{121}$. Skeletonized images of FITC-dextran–labeled vessel networks from 7 zones (a through g; labeled near to far, 0 to 11.7 mm from the periphery of the fibrin graft) were morphometrically analyzed by the method of grid intersection as described in the expanded Materials and Methods. Data were derived from 3 independent CAM experiments per implant formulation. Data are mean±SD. *P$<$0.05 of $\alpha_3$PI$_{1-8}$-VEGF$_{121}$ vs control fibrin alone; **P$<$0.05 of $\alpha_3$PI$_{1-8}$-VEGF$_{121}$ vs VEGF$_{121}$.

**Figure 4.** Comparative, high-magnification analysis of 3-D vascular hierarchy. SEM images show corrosion casts of developing CAM vasculature induced by fibrin implant matrices formulated with freely diffusible native VEGF$_{121}$, or matrix-bound $\alpha_3$PI$_{1-8}$-VEGF$_{121}$. A and B, CAM tissue exposed to implants made of fibrin alone. Regular vasculature was found in the CAM tissue sites adjacent to control implants (A), whereas somewhat reduced capillary density was detected underneath the implant (B). C, E, and G, CAM vasculature induced by release of native VEGF$_{121}$. White stars in C mark dense growth of capillaries into hemangioma-like assemblies. Arrowheads in E denote abrupt changes in vessel diameters on their transition into capillaries. Arrows in G denote sites of vessel splitting and remodeling by intussusception. D, F, and H, Cell-demanded release of $\alpha_3$PI$_{1-8}$-VEGF$_{121}$ resulted in neither perturbation of CAM vascular hierarchy, nor vessel malformation. Connections of arterioles/venules to the capillary bed appear normal (white star in H marks a representative connection). Bars=200 μm (A, B, E, and F); 500 μm (C and D); 50 μm (G and H).
the endothelium appeared homogenous in thickness, with a smooth inner surfaced and a clearly delineated basement membrane (Figure 5F). Contacts between endothelial and periendothelial cells appeared tight.

**Cell-Demanded Release of α_{3}PI_{1-8}-VEGF_{121} Prevents Vessel Leakiness**

We established a quantitative angiogenesis assay in mouse to compare vascular growth and permeability responses to formulations of free or matrix-bound VEGF_{121} in fibrin. For that, porous Teflon chambers filled with 0.55 mL fibrin formulations of free or matrix-bound VEGF_{121} were subcutaneously implanted for 4 days. Due to body irritation by the Teflon chamber, a tissue capsule rapidly formed around the chamber in direct contact with the fibrin gels. Angiogenesis in the newly formed tissue was examined by optical, hematological, and biochemical methods, using ELISA to determine the levels of the endothelial-specific receptors Tie2, VEGFR-2/Flik1, and VEGFR-1/Flt1 (Figure 6); Tie2 level was taken as a measure of endothelial cell/vessel number. Both VEGF variants were comparably effective in enhancing endothelial cell/vessel growth (Figure 6F; \( P<0.05 \) versus fibrin). However, vessel leakiness responses were striking different: tissue exposed to diffusive VEGF_{121} burst showed massive extravasation of blood into the interstitial space (Figure 6B); in contrast, no signs of edema were visible in conditions of α_{3}PI_{1-8}-VEGF_{121} (Figure 6C) or fibrin alone (Figure 6A). Measurements of total hemoglobin content in tissue corroborated these observations. Hemoglobin was increased by diffusible VEGF_{121} by 114% (\( P<0.05 \) versus fibrin), but indifferent versus control fibrin alone in tissue exposed to α_{3}PI_{1-8}-VEGF_{121} (Figure 6D). Local vessel leakage induced by free VEGF_{121} caused drop of systemic hematocrit to subphysiological value, ie, 38.7±0.9% (Figure 6E); hematocrit values remained physiological in experiments with α_{3}PI_{1-8}-VEGF_{121}, or no factor.

VEGF-induced vascularization was associated with increase in Tie2, VEGFR-2/Flik1, but not VEGFR-1/Flt, protein levels in tissue (Figures 6G through 6K). Normalization of VEGF-2/Flik1 to levels of Tie2 did not show any significant change of ratio, ie, comparable numbers of receptor per endothelial cell in any condition (Figure 6I). In contrast, by the same measure, we found a strong, significant decline of VEGF-1/Flt1 protein in endothelial cells in conditions of free VEGF_{121} and some decline under conditions of α_{3}PI_{1-8}-VEGF_{121} (Figure 6J). This response concomitantly translated into a change of distribution between the two VEGF receptors in endothelial cells (Figure 6K). Changes of ratio between VEGF-2/Flik1 and VEGF-1/Flt1 under conditions of diffusive and cell-demanded VEGF_{121} release were 35.8±7.5% and 18.8±5.3%, respectively.
Discussion

In spite of vast knowledge regarding the molecular identity and character of VEGF, as well as its signaling mechanisms, issues remain in knowing how to best use this powerful molecule in therapeutic angiogenesis. Previous studies, as well as ours, demonstrate that VEGF can play either a helpful or a harmful role in tissue vascularization, and that this distinction may depend on the pharmacokinetics of its administration. Our overall concept was to mimic the means by which VEGF is released in vivo: to bind it to ECM components via a mechanism that can be released by cell-associated proteolysis during cell invasion of the matrix. We have mimicked this behavior in fibrin as a therapeutically relevant material platform. We observed potent induction of new vessels in the embryonic chicken CAM by formulations of engineered α5Pls-VEGF121, yielding vessels that were characterized by normal branching morphologies, well-defined lumens, intact basement membranes, and close interactions with periendothelial cells. Studies of vessel permeability in mice validated that cell-demanded α5Pls-VEGF121 release induces integer, nonleaky structures. These features are likely essential for generating lasting and normally functional vascularization.

The VEGF isoform that we studied, VEGF121, lacks heparin-binding character and thus can diffuse relatively freely in the ECM. In mice, the corresponding isoform VEGF120 makes up to 30% of total VEGF protein in certain tissues, suggesting a significant contribution of this molecule.
to angiogenesis. A therapeutic relevance of VEGF121 in improving blood supply has been indicated in clinical trials of adenovirus-mediated VEGF121 myocardial gene therapy. In the chick CAM model of angiogenesis, exogenously applied VEGF121 was found similarly effective as VEGF165 in stimulating endothelial cell proliferation and increasing vascular bifurcation density.

The CAM model of angiogenesis is particularly convenient due to its hierarchical organization, optical transparency, and availability to in vivo imaging techniques for anatomical characterization. The VEGF dosing we explored is similar to doses used in previous studies. The specific doses of VEGF121 formulated in fibrin, ie, 2 μg native VEGF121 and 5 μg α3PI-ε-VEGF121 respectively, were selected by preliminary experimentation according to their ability to induce robust and reproducible angiogenic responses. Doses of 2 to 4 μg VEGF121 or VEGF165 air-dried to Thermanox disc carriers, were also used in previous studies establishing VEGF-mediated angiogenesis in the chicken CAM. The factor XIII–rich formulation used in this study permitted coupling of 90% of the α3PI-ε-VEGF121 within fibrin (Figure 1B). This thus yields a matrix that presented a mixture with 0.5 μg of α3PI-ε-VEGF121, being diffusible, and the remaining 4.5 μg being matrix-bound for cell-demanded release. Control experiments showed that the diffusible α3PI-ε-VEGF121 fraction in this formulation may add to, but does not determine, the response: 0.5 μg diffusible VEGF121 alone in fibrin induced very weak angiogenesis (data not shown), as found also by others, suggesting a threshold dose of VEGF to significantly affect angiogenesis in the CAM model. Furthermore, the angiogenic effects of fibrin matrices grafted after extracting the unbound fraction α3PI-ε-VEGF121 in buffered saline were comparable in quality and quantity to those of unfractionated formulations (data not shown).

Provision of fibrin-bound α3PI-ε-VEGF121 induced structurally integer vasculature, whereas diffusive burst release of native VEGF121 induced malformed, leaky vessel growth. Rapid increase of endogenous VEGF levels is associated with many pathological conditions, eg, ischemia, inflammation, and tumor growth. Endothelial cells express two high affinity receptors of VEGF that are functionally linked, ie, VEGFR-1/Flt-1, but not VEGFR-2/Flk1, serves as the principal signaling receptor in the plasma membrane, and VEGFR-1/Flt1 exists as decoy receptor for VEGF, with minimal signaling activity. In physiological angiogenesis, the net levels of VEGF binding to VEGFR-2/Flk1 are regulated by competing levels of VEGFR-1/Flt1, thereby preventing aberrant activation of VEGFR-2/Flk1. As proposed by Carmeliet and colleagues, perturbation of this balanced distribution of VEGF receptor types could trigger a switch from normal to pathological angiogenesis, eg, in malignancies. Our results are consistent with this idea. Our measurements of cellular VEGF receptor levels showed that VEGFR-1/Flt1 and VEGFR-2/Flk1 were regulated differentially on exposure to exogenous VEGF. A substantial decline of VEGFR-1/Flt-1, but not VEGFR-2/Flk1, was observed, more under conditions of free VEGF121 than of α3PI-ε-VEGF121 (Figures 6I through 6K). We assume that this decline in levels of the decoy receptor VEGFR-1/Flt-1 contributes to aberrant stimulation of VEGFR-2/Flk1, and could explain the superimany, malformed endothelial assemblies on tissue exposure to free VEGF121. Collectively, our morphological and permeability analyses of newly formed vessel structures indicate that cellular proteolytic activity as a temporospatial release trigger for exogenous α3PI-ε-VEGF121 is more compatible with this cellular control of VEGF/VEGFR signaling. It is apparent that the high overall dose of VEGF translated into a low local dose at any point in time when the variant α3PI-ε-VEGF121 was coupled to fibrin and released on cellular demand. In support of this concept, recent studies demonstrated that the microenvironmental amount of VEGF, and not its total dose, determines whether VEGF-induced angiogenesis becomes normal or aberrant.

It is encouraging to us that periendothelial coverage was observed in nascent capillary endothelium that formed under the influence of α3PI-ε-VEGF121 released from fibrin under cellular demand (Figure 5F). Studies of embryonic vascular development have demonstrated the essential role of periendothelial cells for vascular integrity and function. In the absence of a physiological demand, the continued stability of these newly formed vessels will critically depend on continued, balanced presentation of exogenous VEGF. As shown in studies by Keshet and colleagues, VEGF protein levels contributed by the endogenous gene are not sufficient to sustain all vessels generated under conditions of VEGF overexpression; withdrawal of the exogenous VEGF source resulted in selective regression of newly formed, yet immature, vessels devoid of stably associated periendothelial cells by way of disaggregation and apoptosis. Several other classes of morphogens, such as platelet-derived growth factor BB (PDGF-BB), angiopeptin 1, and ephrin-B2, have important roles in stabilizing the newly formed capillaries. Administration of mixed growth factors with complementary activities, eg, VEGF plus angiopoietin 1, or VEGF plus PDGF, may be still more effective in producing a patent and stable vasculature than administration of singular VEGF. Nevertheless, our findings indicate that exogenous VEGF alone, when released slowly in low and sustained dose, may initiate the formation of structurally intact vessels in the target tissue, possibly in concert with endogenous PDGF, angiopoietin 1, or ephrin-B2 activities recruited in this process.

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Expanded Material and Methods

Expression and purification of Vascular Endothelial Growth Factor 121 (VEGF\textsubscript{121})

Native-sequence VEGF\textsubscript{121} was cloned and expressed as histidine-tagged fusion in the bacterial expression plasmid pRSET (Novagen, Madison, WI). The VEGF\textsubscript{121} sequence was generated by PCR using the full-length cDNA of human VEGF\textsubscript{121} (kindly provided by Dr. Weich, National Biotechnology Research Centre, Braunschweig, Germany) using as the forward primer CGCGGATCCGCACCATGGCAGAA (BamHI restriction site underlined) and as reverse primer GGAATTCTCAACCGCTCGCTCTGTC (EcoRI restriction site underlined; stop codon shown in italics). The resulting PCR product was digested with EcoRI and BamHI and ligated to similarly digested pRSET. For VEGF\textsubscript{121} protein expression and purification, the \textit{E. coli} expression host BL21(DE3)pLysS was transformed with the pRSET-VEGF\textsubscript{121} plasmid. The recombinant VEGF\textsubscript{121} was isolated from inclusion bodies, then processed and refolded using a previously published VEGF purification protocol\textsuperscript{1}. Final purification of pure, dimeric VEGF\textsubscript{121} was performed by size exclusion chromatography using a Superdex 200 HR 10/20 column and the Äkta fast performance liquid chromatography system (AmershamBiosciences, Uppsala, Sweden). The homogeneity and identity of VEGF\textsubscript{121} dimer was established by SDS-PAGE and Coomassieblue staining, and TOF-MALDI spectrometry.

Determination of mitogenic activity of matrix-derived VEGFs

The activities of matrix-liberated VEGF forms were determined in human umbilical vein endothelial cell (HUVEC; PromoCell, Heidelberg, Germany) proliferation assays. For these tests, 2 µg of α\textsubscript{2}PI\textsubscript{1-8}-VEGF\textsubscript{121} or VEGF\textsubscript{121} were incorporated within 50 µL fibrin gel matrices. In
the case of \( \alpha_2 \text{PI}_{1-8} \)-VEGF\(_{121} \), an intermediate washing step was performed to remove remaining uncoupled \( \alpha_2 \text{PI}_{1-8} \)-VEGF\(_{121} \). To liberate matrix-conjugated \( \alpha_2 \text{PI}_{1-8} \)-VEGF\(_{121} \) or native VEGF\(_{121} \) into solution, the fibrin gel matrices were solubilized by incubation with 8 mU of the fibrinolytic enzyme plasmin (human plasmin; Sigma, St. Louis, MO). VEGF levels in the lysates were determined by ELISA using DuoSet Human VEGF ELISA kit (R&D Systems, Minneapolis, MN). To assess stimulation of proliferation, HUVECs were incubated with serial dilutions of the VEGF-fibrin lysates in M199 medium supplemented with 10% heat-denatured fetal bovine serum. Three days later, cell proliferation was quantified by colorimetry using a WST-1 reagent assay kit (Roche Diagnostics, Mannheim, Germany).

**Morphometric analysis of the embryonic chicken choriallantois membrane (CAM) vasculature**

Grey scale prints from two still video images per zone were used for the morphometrical estimation of the ‘mean vascular length density’ and the ‘area density of brush-like vascular regions’. The CAM vasculature was skeletonized on grey scale prints and analyzed by the method of grid intersection\(^2\,^3\). Area density was directly obtained by dividing the number of test points falling onto the structure to be measured (e.g. the brush-like areas) by the total number of test points on the reference space\(^2\). Length density was calculated by dividing the number of transsections of the test line with the linear structure to be measured (i.e. the vessels) by the number of test points on the total reference area. The respective formula is \( B_a = \frac{\pi}{2} \cdot \frac{I}{L} \), where \( B_a \) is the length density per area, \( I \) the number of intersections and \( L \) the length of the test line, relative to the area. Since the test line has to be unbiased with respect to structure orientation, a
cycloid line system was used. The test system consisted of 18 sub-sampling tiles containing 1 large (circled) test point and 47 small test points and a cycloid test line of defined length.

The morphometric values were obtained as follows:

1. The brush region area density was assessed by counting hits of small test points on the regions of interest. The result was the ratio of brush region area per reference area in (mm$^2$/mm$^2$).

2. The mean vascular length density was assessed by counting the intersections of the cycloid test line with the blood vessels (mm/mm$^2$).

a. Sub sampling tile of the test system
Mouse subcutaneous tissue-Teflon chamber implant model

A new quantitative angiogenesis assay in mouse was established that permitted to compare vascular growth and permeability responses to formulations of free or matrix-bound VEGF$_{121}$ in fibrin. In this assay, porous Teflon chambers filled with 0.5 mL fibrin formulated with 12 µg VEGF$_{121}$, or α$_2$PI$_{1-8}$-VEGF$_{121}$, or no factor, were subcutaneously implanted for four days. Due to body irritation by the Teflon chamber, a tissue capsule rapidly formed around the chamber in direct contact with the fibrin gels. Angiogenesis in the newly formed tissue exposed to VEGF-fibrin was examined by optical, hematological, and biochemical methods, using ELISA to
determine the tissue levels of the endothelial-specific receptors Tie2, VEGFR-2/Flk1, and VEGFR-1/Flt1.

Preparation of the Teflon chamber implant

Cylindrical, porous tissue chambers made of perfluoro-alkoxy-Teflon (Teflon-PFA, 21 mm x 8 mm diameter, 550 µl volume; Angst&Pfister AG, Basel, Switzerland) and perforated with 80 regularly spaced 0.8 mm holes were used. Both sides of the cylinder were sealed with removable Teflon caps. Four to six Teflon chambers were placed inside a silicon tube container (diameter: 12 mm). One end of the tube was then sealed with silicon rubber. After 24 hr, tubes plus chambers inside were sterilized by autoclaving (121 °C, 15 min). The silicon tubes and along with that the chambers were filled with fibrin gel matrices formulated with VEGF_{121}, α_{2}PI_{1-8}, VEGF_{121} or no factor, under sterile conditions. The final volume of fibrin gel matrix per chamber was 0.55 mL. Each chamber was prepared to contain 12 µg VEGF in fibrin, or fibrin alone.

Subcutaneous implantation of chamber, and retrieval

Six to eight weeks old female white mice (Tif:MAG; provided by the animal breeding facility of NovartisPharma AG, Basel) of 18 to 20 g were used for this assay. The mice received ear markings and were kept in groups (6 animals per cage). Anesthesia of mice was performed by inhalation of 3% isoflurane (Forene Abbott AG, Cham, Switzerland). For subcutaneous implantation, a small skin incision was made at the base of the tail to allow the insertion of an implant trocar. The chamber was implanted under aseptic conditions through the small incision on the back of the animal. The skin incision was closed by wound clips (Mikron Autoclips 9 mm Clay Adams, USA). Four days after, the implants were retrieved from the animals. For that, the animals were anaesthetized with 3% isoflurane and sacrificed using CO_{2}. For determination of hematocrit, blood was collected via the vena cava. Then chambers were retrieved and imaged (a
composite image showing a series of Teflon chamber implants immediately after retrieval is presented below: data described in Fig. 6 of the manuscript were derived from the shown chambers implants). Then the vascularized fibrous tissue capsule formed around each implant was carefully collected for subsequent hematological and biochemical analysis as described below.

Quantification of hemoglobin, Tie2, and VEGF receptors

The fibrous tissue was carefully removed from the Teflon implants, then weighed. Protein lysates of these tissue samples were prepared by homogenization in 2 mL detergent buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 1% NP-40, 20 mM Sodiumfluorid, 1
mM Benzamidin, 15 mM Natriumpyrophoshat, 1 mM phenylmethansulfonylfluorid, 1 mM sodiumorthovanadate) using the a tissue homogenizer (Ultra Turrax T25; Janke & Kunkel, Germany) for 1 min at 24 000 rpm. The detergent lysates were then centrifuged for 1 hr at 7000 rpm. The supernatants were collected, then passed through a 0.45 µm GHP syringe filter (Acrodisc GF, Gelman Sciences, Ann Arbor, Michigan, USA) to remove fatty content. Protein concentrations in the tissue lysates were measured with a BCA protein assay reagent (Pierce; Socochim, Lausanne, Switzerland) and colorimetry in microplates. Hemoglobin content in the supernatants was determined using a Drabkin reagent kit (Sigma, St. Louis, MO), commercial hemoglobin as a calibration standard (Sigma hemoglobin #525, Sigma Chemical Co. Ltd, Poole, Dorset, England), and performing the spectrophotometry at 540 nm. 100 µl aliquots of filtered lysate were assayed after mixing with 0.9 ml of Drabkin’s solution and incubation for 15 minutes at room temperature.

Levels of the endothelial receptors VEGF-R2/Flk1 or VEGF-R1/Flt1 were determined using commercial ELISA kits (Quantikine M for mouse sVEGF R1; Cat. No. MVR100; and Quantikine M for mouse sVEGF R2; Cat. no. MVR200; both from R&D Systems). For determination of Tie2, a new ELISA was established (see below).

**ELISA for Tie2**

A new Sandwich ELISA was established for measurements of Tie2 in tissue lysates. For that, Nunc Maxisorb 96-well plates were incubated with 0.1 mL of anti-Tie-2 capture antibody clone33 (Upstate; LucernaAG, Luzern, Switzerland) used at a concentration of 2 mg/mL, overnight at 4°C. After three washes with 0.2 mL phosphate buffered saline (PBS) containing 0.05% Tween 20 (TPBS), the wells were blocked with 3% Top-Block (JuroAG, Lucerne,
Switzerland) for 2 hr at room temperature. After three washes in TPBS, wells were incubated with 0.2 mL protein lysate (0.1 – 0.5 mg protein) per well for 2 hr. Bound Tie2 protein was detected by 1 hr incubation with a mixture of goat polyclonal anti-Tie2 antibody (R&D Systems, Cat. no. AF762) at a final concentration of 0.5 µg/ml, and alkaline phosphatase conjugated anti-goat mAb (Sigma Cat. no. A-8062) at a final dilution of 1:6000. After washing, bound alkaline phosphatase was measured by colorimetry using p-nitrophenyl phosphate (Sigma) as substrate and reading absorbance at 405 nm. Dilutions of the recombinant human Tie2 extracellular domain fused to the Fc region of human IgG1 (sTie-2Fc) in RIPA buffer were used for calibration (0.1 – 300 ng/well).

References

