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The role of actively released fibrin-conjugated VEGF for *VEGF receptor 2* gene activation and the enhancement of angiogenesis

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Abstract

A major challenge for therapeutic delivery of angiogenic agents such as vascular endothelial growth factor (VEGF) is to achieve sustained, low dose signaling leading to durable neovessel formation. To this end, we recently created a variant of $VEGF_{121}$, TG-$VEGF_{121}$ that directly binds to fibrin and gets released locally in proteolysis-triggered manner. Here we combined noninvasive biophotonic monitoring of *VEGF receptor 2* gene activation in transgenic *VEGFR2-luc* mice and histomorphometry to compare endothelial activation and long-term neovascularization by actively released TG-$VEGF_{121}$ *versus* passively released, diffusible wild-type $VEGF_{121}$ in subcutaneous fibrin implants. Monitoring in real-time over 3 weeks of luciferase signal driven by the *VEGFR2* promoter revealed endothelial activation in skin exposed to wild-type $VEGF_{121}$, but no detectable elevation over fibrin alone by TG-$VEGF_{121}$. Histology at 3 weeks, however, demonstrated that TG-$VEGF_{121}$ promoted vessel growth significantly more effectively and reliably than wild-type $VEGF_{121}$. The majority of vessels surviving to 3 weeks contained stabilizing smooth muscle cells. Yet, by 6 weeks, no extra vessels induced by exogenous VEGF were left. In conclusion, release of fibrin-conjugated variant TG-$VEGF_{121}$ elicited lower *VEGFR2-luc* activation than wild-type $VEGF_{121}$ yet significantly more vascularization. In the absence of true physiological demand, even stabilized vessels are ultimately regressed.
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1. Introduction

Preclinical trials have indicated that administration of vascular endothelial growth factor (VEGF) as protein [1], gene [2,3], or factor-overexpressing cell transplants [4] can stimulate resident endothelial cells to form new capillary tubes, thereby improving regional blood flow in ill-perfused adult tissues. Yet, VEGF delivery to patients has been disappointing [5,6]. It is not clear whether initial angiogenic response triggered by exogenously delivered VEGF will provide long-term benefit for blood flow recovery at the disease treatment site: immature, leaky vessel formation, aberrant vessel growth, and vessel regression present serious complications. The lack of lasting angiogenesis observed in many therapeutic trials is due to difficulties in achieving an appropriate dosage of VEGF, a growth factor with an apparent narrow therapeutic window [4,7]. Implantation of clonal populations

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of genetically engineered myoblasts secreting different discrete amounts of VEGF, has suggested the existence of lower and upper thresholds of microenvironmental VEGF concentrations for induction of safe and long-term vessel growth [4,7–9]. An important implication of these studies is that local, microenvironmental dose, not total dose of VEGF in tissue determines safety and efficacy in a therapeutic setting. Further, conditional VEGF overexpression in adult mice revealed that newly formed vessels only survive when exogenous VEGF signal is supplied over several weeks to allow for their maturation into stable vessels enwrapped with supporting pericytes or smooth muscle cells [10,11]. Early deprivation of exogenous VEGF signal results in selective ablation of newly formed, immature vessels by way of apoptosis and disaggregation [12]. Bolus injection of soluble VEGF protein into the circulation or directly into the ischemic zone fail to achieve the prolonged supply of physiological low dose of VEGF that is necessary to produce a mature, lasting vascular network, and potentially lead to adverse side effects such as hemorrhage or hypotension. Together, exogenously induced angiogenesis in the adult demands tight regulation.

Multiple studies in different laboratories have indicated that incorporation of growth factor signal into slow release polymer formulations could present a means for better control of dose, location and duration of active signal in tissue, as reviewed in Refs. [13,14]. Our laboratory has pursued biomimetic schemes of VEGF delivery from biopolymer cell ingrowth matrices that prevent diffusion of VEGF from the matrix but permits its sustained release under the control of matrix-degrading enzymes locally secreted by cells at the treatment site [15,16]. In the body, the prevalent form of VEGF in tissue, $VEGF_{165}$, physically binds to heparan sulphate proteoglycans in the extracellular matrix (ECM), which protects the growth factor from clearance and degradation until released by local cellular enzymatic activity. We recently introduced a molecular variant of VEGF, TG-$VEGF_{121}$, that follows this concept of ECM-binding and cell-mediated release in the natural wound healing matrix fibrin, which is clinically widely applied as 'fibrin glue' tissue sealant [15,17]. TG-$VEGF_{121}$ spontaneously cross-links to fibrinogen by the transglutaminating activity of factor XIII during fibrin polymerization. Covalently tethered TG-$VEGF_{121}$ is protected from diffusion, contrary to wild-type $VEGF_{121}$ admixed into fibrin, which diffuses out rapidly [15]. In TG-VEGF–fibrin gels, gradual degradation of the bulk matrix fibrin by local fibrinolytic activities such as plasmin or matrix metalloproteinases results in concomitant, local liberation of low levels of TG-$VEGF_{121}$ into tissue. Our previous analysis of acute angiogenic response, i.e. few days post-implantation, in different experimental animal models showed that fibrin-conjugated TG-$VEGF_{121}$ produced more and at the same time structurally more normal vessels than wild-type $VEGF_{121}$, and avoided vascular leakage [15].

In the present investigation, we looked at durable vessel formation in response to passive *versus* active release of VEGF. Fibrin gel implants formulated with wild-type $VEGF_{121}$ and TG-$VEGF_{121}$ presented a means to realize passive and active VEGF release, respectively. Our first analysis concerned endothelial activation. VEGF exerts its biological activities for endothelial cells through two related receptor tyrosine kinases, namely VEGF receptor 2 (VEGFR2; Flk-1/KDR) and VEGF receptor 1 (VEGFR1/Flt-1); where VEGFR2 acts as the principal signaling receptor [18]. Using surgical implantation into transgenic *VEGFR2-luc* mice [19], we could monitor angiogenesis response at the implantation site in real-time over 3 weeks via the luciferase reporter signal driven by the promoter of the *VEGFR2* gene. Our second analysis concerned histological vessel growth at the implantation site at 3 and 6 weeks.

2. Methods

2.1. Vascular endothelial growth factors

Preparation of *E.coli* recombinant proteins TG-$VEGF_{121}$ and $VEGF_{121}$ has been described previously [15,17].

2.2. Fibrin gel matrices formulated with VEGF

Fibrin gel matrices were prepared, as described previously [15], by mixing the following components at the final concentrations of 10 mg/mL fibrinogen (Fluka AG, Buchs, Switzerland), 2 U/mL factor XIII (provided by Baxter Bioscience AG, Vienna), and 2.5 mM $CaCl_2$; TG-$VEGF_{121}$ or $VEGF_{121}$ were added to the fibrinogen solution prior to initiation of fibrin gelation by adding 2 NIH units/mL gel matrix of human thrombin (Sigma, St. Louis, MO).

2.3. Implant model

The investigation conforms to 'The Guide for the Care and Use of Laboratory Animals' published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Further, all animal experiments were done in strict accordance with the Swiss law for animal protection and approved by the Governmental Veterinary office of the State of Basel (license number 1325). *VEGFR2-luc* mice [FVB/N-Tg *(Vegfr2-luc)*Xen] (Xenogen Corporation, Alameda, CA) of 18–20 g were used. These mice carry a transgene containing a 4.5-kb murine *VEGFR2* promoter fragment that drives the expression of the firefly luciferase reporter protein [19]. Implants consisted of custom made sterile cylindrical silicon cups of 8-mm outer diameter and 5-mm inner diameter which were filled with 0.25 mL fibrin gel matrix containing 12.5 μg $VEGF_{121}$ or TG-$VEGF_{121}$. Control cups were filled with fibrin alone. Anesthesia of mice was performed by inhalation of 3% isoflurane (Forene® Abbott AG, Cham, Switzerland). For subcutaneous implantation, a small skin incision was made above the spine and the cups were implanted under aseptic conditions through the incision on the back of the animal. The cups were placed such that the fibrin matrix was in contact with the subcutaneous layer of the mouse skin. The skin incision was closed with wound clips (Mikron Autoclips, 9 mm, Clay Adams, USA). Each mouse received two implants, a VEGF-releasing implant on one side, and a control implant, fibrin alone, on the contralateral site.

2.4. Bioluminescent in vivo monitoring of VEGF receptor 2 gene activation

Tissue luciferase activity at the implant sites was recorded on post-implantation days 2, 5, 8, 12, 15, and 19 using an IVIS 100 biophotonic imaging system (Xenogen Corporation). A day 0 image was taken immediately following implantation. For imaging, mice were anesthetized with 3% isoflurane, then 150 mg/kg luciferin (Biosynth, Staad, Switzerland) was injected intraperitoneally. Photons were recorded for 5 min starting 15 min after luciferin injection in a light-tight specimen chamber using a cooled charge-coupled device camera. Emitted photons from the manually selected region of interest

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