Vascular Regeneration by Local Growth Factor Release Is Self-Limited by Microvascular Clearance

Kha N. Le, PhD; Chao-Wei Hwang, MD, PhD; A. Rami Tzafriri, PhD; Mark A. Lovich, MD, PhD; Alison Hayward, DVM; Elazer R. Edelman, MD, PhD

Background—The challenge of angiogenesis science is that stable sustained vascular regeneration in humans has not been realized despite promising preclinical findings. We hypothesized that angiogenic therapies powerfully self-regulate by dynamically altering tissue characteristics. Induced neocapillaries increase drug clearance and limit tissue retention and subsequent angiogenesis even in the face of sustained delivery.

Methods and Results—We quantified how capillary flow clears fibroblast growth factor after local epicardial delivery. Fibroblast growth factor spatial loading was significantly reduced with intact coronary perfusion. Penetration and retention decreased with transendothelial permeability, a trend diametrically opposite to intravascular delivery, in which factor delivery depends on vascular leak, but consistent with a continuum model of drug transport in perfused tissues. Model predictions of fibroblast growth factor sensitivity to manipulations of its diffusivity and transendothelial permeability were validated by conjugation to sucrose octasulfate. Induction of neocapillaries adds pharmacokinetic complexity. Sustained local fibroblast growth factor delivery in vivo produced a burst of neovascularization in ischemic myocardium but was followed by drug washout and a 5-fold decrease in fibroblast growth factor penetration depth.

Conclusions—The very efficacy of proangiogenic compounds enhances their clearance and abrogates their pharmacological benefit. This self-limiting property of angiogenesis may explain the failures of promising proangiogenic therapies. (Circulation. 2009;119:2928-2935.)

Key Words: angiogenesis ■ collateral circulation ■ growth substances ■ ischemia ■ microcirculation ■ pharmacokinetics

Stimulation of neovascularization with angiogenic growth factors might reduce myocardial infarct size and improve cardiac function1 and peripheral tissue2 perfusion. Yet, impressive results in tissue culture and animal studies3-7 have not been sustained in clinical trials.8-11 Intravascular delivery of angiogenic factors is convenient but challenged by the requirements for high doses and long residence times.8,10 Cell and gene injections might provide a continuous source of growth factor, and intramyocardial or pericardial delivery has elevated local tissue drug concentrations with lower systemic exposure in animal models.12 However, the promise of symptomatic improvement and increased capillary density seen in early clinical trials with intramyocardial injections of fibroblast growth factor (FGF) after coronary artery bypass grafting13 has not endured in larger clinical trials, and clinical outcomes in general with local growth factor delivery have been mixed.9,14-18

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Some have postulated that myocardial growth factor concentrations, drug residence time, or both were inadequate for sustained angiogenesis despite controlled release delivery.19 However, the local pharmacokinetic processes governing the uptake and distribution of growth factors in highly vascularized tissues such as myocardium remain ill defined. It is therefore possible that the reverse is true and that growth factor concentrations were actually more than sufficient initially. We hypothesized that local delivery can induce neocapillary growth but, in doing so, changes the balance between drug delivery and microvascular drug clearance to favor the latter, so that the very pharmacological efficacy of these compounds limits their biological effect.

We used a series of ex vivo analytical and in vivo experimental animal models to quantify how growth factors
distribute within vascularized tissue and how neovascularization after delivery of angiogenic growth factors affects their pharmacokinetics and efficacy. These studies strongly suggest that angiogenesis is powerfully self-regulating in that the capillaries induced by angiogenic drug therapy may increase clearance rates, limiting tissue levels of growth factor and subsequent angiogenesis even with sustained delivery.

Methods

Ex Vivo Myocardial Drug Delivery With and Without Perfusion

Sprague Dawley rats (0.5 to 0.6 kg) were anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine and anticoagulated with 1000 U heparin SC before to CO2 euthanasia. The aorta was cannulated and the heart was retrograde perfused with cardioplegia (osmolality, 289±5 mOsm/kg H2O) composed of Krebs-Henseleit buffer (Sigma-Aldrich, St Louis, Mo) with high potassium (30 mmol/L KCl) and 4% BSA (Sigma-Aldrich) to establish diastolic arrest. The heart was excised and perfused at 95 mm Hg. Coronary flow was monitored periodically and ranged from 8 to 10 mL/min throughout the experiment. The perfusate was oxygenated by a foam bubble oxygenator with 95% O2/5% CO2 at 37°C. Samples were also examined in the absence of coronary perfusion to eliminate other effects. Here, the aorta was cannulated and flushed with perfusate. As blood cleared from the circulation, coronary outflow from the coronary sinus was stopped by clamping the right atrium and pulmonary artery, ensuring the patency of myocardial capillaries and ensuring that the only difference between the control and perfused cases was coronary perfusion. The entire configuration resided within an enclosed box with 100% humidity. We ascertained myocardial viability at 6 hours by quantitatively documenting no additional tissue edema on hematoxylin and eosin–stained sections. Experimental protocols were in accordance with National Institutes of Health guidelines for the humane care and use of laboratory animals and the MIT Committee on Animal Care.

Rabbits (New Zealand White, 3 to 3.5 kg) received an intramuscular injection of 35 mg/kg ketamine and 5 mg/kg xylazine, inhaled isoflurane anesthesia (1% to 3%), and positive pressure ventilation via a 3.0-mm endotracheal tube. The chest was shaved and sterilly prepared with Betadine and alcohol. A left thoracotomy was performed after local lidocaine injection. A clamp kept the chest open, and a small opening in the pericardium was created with care to minimize pericardial damage. The left anterior descending coronary artery was ligated. Ischemia was confirmed by ST-segment elevation on simultaneous continuous ECG. Sodium alginate polymeric de-

Table. Continuum Pharmacokinetic Model Equations for Epicardial Drug Delivery

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
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| Equation 1 | \[
\frac{\partial C}{\partial t} - D \frac{\partial^2 C}{\partial x^2} = -kC
\] |
| Equation 2 | \[k = \left(\frac{2 \pi}{R_{mv}}\right) \frac{P_{mv} \phi_{mv}}{1 - \phi_{mv}}\] |
| Equation 3 | \[\frac{C}{C_0} \approx \text{erf} \left(\frac{x}{2\sqrt{Dt}}\right)\] |
| Equation 4 | \[\%\text{clearance} = 100 \times \left(1 - \frac{M_{\text{diff,clear}}}{M_{\text{diff}}}\right)\] |
| Equation 5 | \[\%\text{clearance} = 100 \times \left(1 - \frac{M_{\text{diff,clear}}}{M_{\text{diff}}}\right)\] |

Detailed derivations are included in the Methods section of the online-only Data Supplement. Equation 1 describes the transport of drug in the presence of capillary clearance. C represents the drug concentration in the tissue as a function of time t, distance from the epicardium x, diffusivity D, and apparent clearance constant k. Equation 2 relates the apparent clearance constant k to capillary permeability Pmv, capillary volume fraction, and capillary diameter Rmv. Equation 3 describes steady-state tissue concentration normalized to source concentration, C0, in the absence of capillary perfusion. Equation 4 shows the steady-state tissue concentration in the presence of capillary perfusion, where \(\ell\) is drug penetration depth. Equation 5 shows the percentage of drug clearance by capillary perfusion as a function of k and D.

Quantification of In Vivo FGF1 and Blood Vessel Distribution

Two frozen myocardial cores were excised adjacent to the polymeric devices with an 8-mm-diameter biopsy punch (Miltex, York, Pa). Tissue cores were cryosectioned (Leica CM1850, Leica, Germany) perpendicular to the epicardium for quantitative epifluorescence imaging (Leica DMRA2 microscope; Hamamatsu C4742–95 camera, Bridgewater, N.J.; MetaMorph software, Sunnyvale, Calif; Texas Red filter set). Because the fluorescent intensity of TR-FGF2 is linearly proportional to the distance from the source to 90% drop-off threshold, as \(x_{90} = \ell \ln(10)\). The other tissue core was cryosectioned into 10-μm-thick sections in the transmural direction. Sections were fixed for 5 minutes with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pa), washed for 30 seconds with cold acetone (Sigma-Aldrich, St Louis, Mo), incubated in blocking serum (200 μL 1% chicken serum for 1 hour at 37°C) and then in goat anti–PECAM-1 IgG (Santa Cruz Biotechnology, Santa Cruz, Calif; 200 μL of 1:50 dilution for 2 hours at 37°C), washed 3 times in 0.1% Tween 20 in PBS, incubated again with Alexa Fluor 488 chicken anti-goat IgG (Invitrogen, Carlsbad, Calif; 200 μL of 1:200 dilution for 2 hours at 37°C), washed 3 times in Tween 20/PBS, coverslip mounted, and imaged immediately with a fluorescence microscope (Leica DMRA2, FITC filter set). The images were thresholded to maximize the signal-to-noise ratio with Matlab (MathWorks, Natick, Mass), yielding binary images of vessel distribution. Neovascular formation of tissue regions within 500 μm from the epicardial source was quantified by computing the tissue area fraction stained by

\[
\text{clearance} = 100 \times \left(1 - \frac{M_{\text{diff,clear}}}{M_{\text{diff}}}\right)
\]
PECAM-1 (total number of pixels with PECAM-1 stain divided by the total number of pixels of tissue area).

Statistical Analysis
All data are presented as mean±SEM, except values for the clearance rate constants (k), which are reported as mean±propagated SE. Propagated errors, $s_k$, were calculated with this formula:

$$\left(\frac{s_k}{K}\right)^2 = \left(\frac{s_D}{D}\right)^2 + 2 \left(\frac{s_{x_{90}}}{x_{90}}\right)^2,$$

where $s_D$ and $s_{x_{90}}$ are SEs for effective diffusivity, D, and penetration depth, $x_{90}$, respectively. Statistical analyses were performed with the Student $t$ test when appropriate. Values of $P<0.05$ (2 tailed) were considered statistically significant. Nonlinear regression was performed with GraphPad software (Prism 5) to fit steady-state spatial drug distributions to Equations VI and XI in the online-only Data Supplement in obtaining values for the clearance rate constant and effective diffusivity, respectively.

Results
FGF Distribution Is Limited by Myocardial Perfusion
Drug transport through and deposition within tissues are governed by molecular weight–dependent processes such as diffusion and convection and physicochemical attributes such as binding, partitioning, and metabolism.21–23 We examined the effects of capillary perfusion on myocardial growth factor transport in rat hearts incubated at constant epicardial source concentrations (Figure I in the online-only Data Supplement) with and without controlled coronary flow. When delivered to the ex vivo myocardium in the absence of flow, TR-FGF2 distributed via diffusion to a penetration depth of 66 μm in 6 hours (Figure 1). Restoration of coronary perfusion reduced TR-FGF2 penetration depth >2-fold to 28 μm, localizing growth factor closer to the epicardial drug source (Figure 2).

Drug Diffusivity, Transendothelial Permeability, and Vessel Density Influence Local Drug Distribution and Deposition
We examined the impact of coronary flow on drug penetration within the context of a continuum pharmacokinetics model of drug diffusion in the face of microvascular clearance (Equation 1 in the Table). In the absence of perfusion, the growth factor distribution curve mimics the analytical solution of the diffusion equation (Equation 3 in the Table) with an apparent diffusivity of 0.02±0.001 μm²/s for TR-FGF2. This value is 4 orders of magnitude smaller than the reported diffusivity of FGF in free aqueous solution,24 reflecting the impact of steric hindrance and binding within tissues. TR is small (600 Da) and hydrophilic. Its diffusion is significantly higher than FGF in heparin sulfate proteoglycan-rich myocardium, and detected fluorescence is likely specific for TR-FGF-2. In the presence of coronary flow, the capacity of capillaries to clear drugs is restored, and the distribution of TR-FGF2 at 6 hours approaches an exponential steady-state profile consistent with an apparent clearance rate constant: $k = 1.15±0.06 \times 10^{-4}$ s⁻¹ (Equation 4 in the Table). Given a normal myocardial capillary density of $\approx 12.9\%$25 and FGF2 aqueous diffusivity of $2.2 \times 10^{-2}$ μm²/s,24 our estimate of the clearance rate constant of FGF2 implies...
that the transendothelial permeability and ratio of permeability to diffusivity are \( \approx 1.9 \times 10^{-3} \) \( \mu \text{m}^2/\text{s} \) and \( 8.8 \times 10^{-6} \) \( \mu \text{m}^{-1} \). This estimate is in line with the transendothelial permeability of a molecule with a molecular size of FGF2.26

Analytic models of drug transport and loss to capillary flow were evaluated across a range of diffusivities, transendothelial permeabilities, and microvascular volume fractions. Steady-state results (Equations VII and IX in the online-only Data Supplement Methods section) were used because equilibrium is rapidly achieved. Penetration depth at steady state increases as the square root of the diffusion coefficient (Figure 2A) and decreases as the square root of the transendothelial permeability constant (Figure 2B). It is worth contrasting our results with those from systemic drug delivery through an intravascular route, in which tissue absorption is proportional to transendothelial permeability. Increasing transvascular penetration in systemic delivery requires drugs that permeate across the endothelium and interventions that increase, rather than decrease, vascular permeability.27–29

Tissues with higher degrees of vascularization, as embodied by the vascular volume fraction, clear drugs faster and have lower steady-state drug penetration (Figure 2C). Thus, steady-state drug penetration and distribution are highly dependent on drug diffusivity through tissue and net microvascular clearance, the compounded effect of transendothelial permeability and microvascular volume fraction (Equation 2 in the Table).

The theoretical reduction in total deposition caused by capillary clearance was calculated (Equation 5 in the Table) and expressed as a function of the clearance rate constant, \( k \) (Equations VIII and XII in the online-only Data Supplement). Percentage clearance of drug with coronary perfusion is most sensitive for clearance rate constants ranging between \( 1 \times 10^{-3} \) and \( 1 \times 10^{-2} \) \( \text{s}^{-1} \) (Figure 3A). Notably, our measurement of the clearance rate constant of TR-FGF2 falls within this range, suggesting that FGF clearance is highly sensitive to transendothelial permeability and microvascular volume fraction.

To examine whether the clearance rate constant of FGF might be modulated by altering its physicochemical properties, we used quantitative fluorescence imaging to contrast the distribution of TR-FGF2 alone or in association with SOS. SOS induces FGF dimerization and increases the effective molecular weight of TR-FGF2.30 The increase in size was confirmed by size-exclusion chromatography and should reduce transendothelial permeability, capillary washout, and effective diffusivity. Indeed, in the absence of coronary perfusion, TR-(FGF2)2-SOS penetrated 40% less than TR-FGF2 into the myocardium (40 \( \mu \text{m} \), with an effective diffusivity of \( 0.013 \pm 0.001 \) \( \mu \text{m}^2/\text{s} \)), reflecting its increased size (\( D_{\text{eff}}^{\text{TR-FGF2}}=0.021 \pm 0.001 \) \( \mu \text{m}^2/\text{s} \); Figure 1 versus 3B).

But, the larger compound also was less affected by coronary perfusion, with penetration depth falling only 26% to 30 \( \mu \text{m} \) and total deposition falling by only 12% (Figure 3B). The muted sensitivity of TR-(FGF2)2-SOS to flow is consistent with the estimated clearance rate constant (\( k=4.37 \pm 0.33 \times 10^{-5} \) \text{s}^{-1} ), model predictions (Figure 3A), and our hypothesis that larger molecules enter capillaries less readily.

**Figure 3.** FGF distribution is sensitive to alteration in drug clearance. A, Percentage of drug cleared by capillaries calculated using the analytical model (Equation 5 in the Table) as a function of clearance rate constant, \( k \) (black line). Experimental data points for TR-FGF2 and TR-(FGF2)2-SOS analyzed by Equation M1 are superimposed (magenta squares) on model predictions providing perspective on the sensitivity of FGF2 to manipulation of its clearance constant. B, Distribution and representative fluorescence microscopy images of TR-(FGF2)2-SOS in rat myocardium with coronary (magenta) and without (blue) coronary perfusion. Data represent mean \( \pm \) SEM (n=3).

**In Vivo Angiogenic Response Limits Drug Distribution**

Conventional pharmacokinetic models for drug distribution do not take into account the potential that drugs can alter capillary density, transendothelial permeability, or drug clearance. Yet, angiogenic growth factors such as FGF1 and FGF2 are specifically administered to induce capillary growth, and it would be unreasonable to assume that transendothelial permeability and drug clearance are not similarly modified. Induced neovascularization implies an increase in the density of blood capillaries that could provide negative feedback, limiting growth factor tissue penetration (Figure 2C). Our analysis of myocardial drug transport suggests that FGF would be particularly sensitive to induced capillary washout (Figures 1 and 3A). We tested this hypothesis in vivo using radiolabeled FGF1 (\(^{35}\text{S}-\text{FGF1}\)). TR-FGF2 could not be used in vivo because its labeling intensity is much lower than that of \(^{35}\text{S}-\text{FGF1}\), rendering it virtually transparent at the doses delivered. Notably, ex vivo experiments with \(^{35}\text{S}-\text{FGF1}\) were well explained by the diffusion-clearance model and suggest that the transport parameters estimated for TR-FGF2 are similar to those of \(^{35}\text{S}-\text{FGF1}\) (online-only Data Supplement). Heparin-bound biologically active \(^{35}\text{S}-\text{FGF1}\) fractions were isolated, incorporated into heparin Sepharose-alginate wafers that sustain-release FGF1 for over 30 days in vitro and in vivo.
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Figure 4. Polymeric devices sustain release FGF1 over 30 days. A, Cross-sectional area of hematoxylin and eosin–stained rabbit myocardial tissue shows location of drug release source (adjacent to left ventricular free wall) and interface between drug source and myocardium. B, Percentage of cumulative 35S-FGF1 released into PBS buffer in vitro (○; data represent mean±SEM [n=10]; 100% corresponds to ~90 μg 35S-FGF1 per circular disk of 8-mm diameter and 1-mm thickness). Throughout the in vivo experiment (●; data represent mean±SEM; n=3), 100% corresponds to ~450 μg 35S-FGF1 per device (20×20×1 mm), calculated as follows: percent drug released=(total drug within source– total drug remaining)/total drug within source×100%.

Figure 4B, and implanted in the pericardial space of ischemic rabbit hearts (Figure 4A). FGF1 successfully penetrated 442±91 μm into the myocardium over the first 2 days after release initiation. Yet, despite sustained delivery, penetration regressed over time, falling 5-fold by day 8 (81±30 μm), and remained low through day 31 (Figure 5A). Enhanced growth factor clearance was associated with the induction of neovascularization. Two days after device implantation, the fraction of PECAM-1–stained tissue was 56% greater in animals receiving FGF1 (4.4%) compared with baseline density (2.8%) in control animals with identical devices devoid of growth factor (P<0.05; Figure 5C). Neovascularization peaked at day 8 (8.7%), double that from day 2 (P<0.01; Figure 5C), coinciding with the drop in drug distribution. Similarly, the fraction of PECAM-1–stained tissue decreased significantly from day 8 to 31 (−62%; P<0.01; Figure 5C), most likely from regression of neocapillaries as the concentration of FGF1 becomes subtherapeutic. Examination of drug delivery devices for remaining drug content confirmed that the decreasing myocardial concentrations arose from increasing capillary clearance rather than decreasing drug delivery. None of the delivery devices were depleted of drug, and all continued to release drug with constant flux (Figure 4B) after the expected burst release of ~37.5% during the first 6 hours.

Discussion

The potential of angiogenic promoters of endothelial cell growth and neovascularization is well established in cell and tissue culture.31,32 However, angiogenic growth factors have yet to produce stable sustained angiogenesis in clinical trials.8,10 Although there are undoubtedly biological factors involved,33 our study suggests that fundamental transport barriers, particularly the self-limiting pharmacokinetics of angiogenic therapy, also may impair the realization of sustained neovascularization.

Our results reveal that drug transendothelial permeability plays a vital role in governing drug distribution. Lower transendothelial permeability ensures greater myocardial drug penetration after local application (Figure 2B). FGF is an arterial vasodilator, but this cannot explain our results because expected changes in arteriolar volume34 could not reach the scale of our results or explain the difference in growth factor distribution between perfused and static flow conditions, both of which saw growth factor. The dependence of drug distribution on transendothelial permeability and capillary density takes on further complexity for angiogenic compounds that can remodel their tissue environment. Over the course of treatment, vessel density in ischemic tissues increases in FGF1-Iaden tissue regions (within 500- to 600-μm depth from epicardium; Figure 5B). Indeed, abundant neovascularization occurs from day 2 to 8, but drug washout rises as well, precipitously dropping the local tissue concentration of FGF1 (Figure 5A) and penetration depth of FGF1 despite no quantifiable change in growth factor delivery. The instability of FGF1 in the absence of heparin35 cannot account for the observed effects because the factor was released directly from the heparin Sepharose devices to the epicardial tissue. Released FGF1 likely binds reversibly to myocardial heparin sulfate proteoglycans, prolonging its tissue half-life. Indeed, angiogenic activity was observed 8 days after delivery (Figure 5). Moreover, changes in angiogenic action correlated with regression of FGF1 distribution, not a decrease in activity or stability. Continuum pharmacokinetics (Equation XV of the online-only Data Supplemental Methods) suggest a 5-fold increase in capillary washout between day 2 and 8. Such an increase in the FGF1 clearance could arise from an increase in microvascular density and/or transendothelial permeability (Equation 2 in the Table). The observed 65% rise in capillary density between days 2 and 8 indicates that the latter plays a dominant role and suggests that the induced vasculature is immature and highly permeable to FGF1.

Our findings, therefore, offer possibilities for engineering drugs to penetrate tissue better by reducing their transendothelial permeability. FGF2 lies within the region where drug penetration depth is highly sensitive to the clearance rate constant, k (Figure 4A), which is directly proportional to transendothelial permeability (Equation 2 in the Table), whereas (FGF2)2-SOS, with its 2.6-times-lower clearance, is less affected by flow. More than 50% of FGF2 but only 12% of (FGF2)2-SOS is cleared by coronary perfusion (Figure 1B versus 3B). Therefore, one way to decrease permeability is to consider drugs of higher molecular weight. Although higher molecular weight implies lower myocardial drug diffusivity, the ratio of permeability to diffusivity can drop 2 orders of magnitude as molecular radius increases from 2.4 to 36 Å.26 A different method for modulating transendothelial permeability is by modification of drug charge. Indeed, it has been shown that negatively charged dextrans exhibit 10-times-
lower transendothelial permeability than neutral analogs. This approach may present a method for lowering transendothelial permeability of a drug to increase penetration depth and deposition.

Our study also highlights the impact of capillary clearance on myocardial drug distribution and elimination and sets a ceiling for the depth of drug penetration at steady state. Such clearance is dependent on the transendothelial permeability of the delivered drug and tissue vascularity. At normal arterial $P_O_2$, a significant portion of capillaries are relatively constricted. Normal myocardium can respond swiftly to arterial hypoxia by dilating capillaries and effectively decreasing the intercapillary distance and thus the oxygen diffusion barrier. Through reactive hyperemia, the glyocalyx lining of endothelial cells can be modified by reactive oxygen species to increase transendothelial permeability and capillary and postcapillary venule diameter. However, myocardial capillary density declines markedly with ischemia and infarction, limiting capillary reserve. Because capillaries act as spatially distributed sinks to drug, myocardium with denser functional vascularity will clear drug more efficiently and rapidly, creating zones of low drug concentration. Gradation in vascularity in diseased tissue thus has important consequences for local drug delivery. Because capillary flow in ischemic and infarcted regions is substantially lower than in normal tissue, our data predict that drug penetration would be greater. Placing delivery devices directly in the ischemic region should confer the pharmacokinetic advantages of decreased capillary washout; in well-perfused regions, drug molecules may never reach ischemic areas at sufficient levels. Pericardial delivery of growth factors to target endocardial ischemic regions may thus prove futile because drugs will not easily cross well-perfused epicardial regions. Indeed, drug penetration in the presence of capillary perfusion was limited ex vivo (Figure 1) and in vivo (Figure 5) despite ample time for distant diffusion.

The integrated studies presented suggest that angiogenesis is powerfully self-regulating in that the very capillaries induced by angiogenic drug therapy may increase clearance. The pharmacodynamic changes during the early angiogenic therapy can tip the pharmacokinetics to conditions that are unfavorable for growth factor penetration, which in turn affects long-term therapeutic goals. This mechanism implies a natural upper limit effect for pharmacological revascularization, which restricts angiogenic drug penetration and spatially confines the sprouting of new vessels near the drug source. At day 31, the FGF1 level in the 100- to 500-μm tissue region falls to undetectable levels, and significant regression of neovascularization consequently occurs in the absence of local growth factor (Figure 5C). One might well imagine that such forces are essential to endogenous regulation of tissue morphogenesis and repair and that loss of such regulation may help explain the growth of vascular tumors and other arteriovenous malformations and anomalies.

The interdependence of the pharmacokinetics and pharmacodynamics elucidated in this study may explain the diffi-
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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Proangiogenic growth factors have long been considered for regenerating ischemic tissues. Yet, sustained clinical benefit has not followed promising preclinical findings even with advances in local delivery technology. Quantitative and animal models suggest that the limited late efficacy of local delivery of angiogenic factors stems partly from the very early success at inducing vessel growth. Fibroblast growth factor released from a polymeric implant penetrates ischemic rabbit myocardium in vivo for the first 2 days of local delivery and indeed induces neovascularization. But, as new vessels are formed, drug is cleared rapidly, and the induced vessel mass regresses. As each wave of new vessels appears in response to the angiogenic factor pools, the factors are cleared, and the angiogenic signal abates as a receding wave that implodes back toward the growth factor source. Despite a constantly eluting source of fibroblast growth factor, the front of new vessels continues to fall back. Eight days after release initiation, neovascularization remains but only as a regressed zone immediately around the delivery device. By day 30, there is only a superficial layer of vessels around the release device. This interdependence of pharmacokinetics and pharmacodynamics may explain the difficulty of realizing sustained clinical angiogenesis with local release and suggests that efficacy depends on device placement and drug transendothelial permeability. The quantitative framework presented here may help guide rational selection of specific angiogenic compounds on the basis of a favorable physicochemical profile and drug delivery strategies that take advantage of the tight regulation between growth factor pharmacokinetics and angiogenic pharmacodynamics.
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Supplemental Materials

Supplemental Methods

Measurement of FGF in Outflow

We verified that capillary washout of FGF through microvascular clearance was indeed responsible for the reduction in FGF penetration in the presence of perfusion with \(^{35}\text{S}\)-FGF1. Rat hearts were isolated and perfused using identical procedures and experimental parameters described in the “Ex-vivo Myocardial Drug Delivery” methods section for perfused hearts. \(^{35}\text{S}\)-FGF1 (4.2 mg/ml) was applied to the epicardial surface (n=3) for 3 hours. The perfusate in these experiments was not recirculated, hence presence of \(^{35}\text{S}\)-FGF1 in the outflow results from direct washout of exogenous growth factor in tissue. Outflow perfusate was collected in 6-30m fractions. At the end of perfusion experiments, 3ml samples of the outflow fractions were decolorized with 0.5ml hydrogen peroxide at 60°C for 1 hour (30%, Sigma-Aldrich), and assayed for \(^{35}\text{S}\) radioactivity using liquid scintillation counter (Packard).

Continuum Pharmacokinetic Model of Myocardial Drug Transport

Following Tzafriri et. al. \(^1\) we model interstitial drug transport in a perfused tissue using the classical diffusion equation with a linear sink term

\[
\frac{\partial C}{\partial t} - D \frac{\partial^2 C}{\partial x^2} = -kC
\]

(S1)

where the apparent clearance rate constant \(k\) is proportional to trans-endothelial permeability as

\[
k = \frac{P_{mv} S_{mv}}{(1 - \phi_{mv})}.
\]

(S2)
Here $S_{mv}$ is the surface fraction of capillaries and $\phi_{mv}$ is the capillary volume fraction. Since the surface fraction of a cylinder of radius $R_{mv}$ is related to the volume fraction as $S_{mv} = 2\phi_{mv} / R_{mv}$, we can rewrite the proportionality between the apparent clearance rate constant and trans-endothelial permeability as

$$k = \left( \frac{2}{R_{mv}} \right) \frac{P_{mv} \phi_{mv}}{1 - \phi_{mv}}. \quad (S3)$$

A rich literature exists on the analysis of Eq. S1. For our purposes, it suffices to note that localization of the experimental growth factor profiles close to the drug source justifies the analysis of distribution profiles in terms of penetration into a semi-infinite domain. Namely, we assume that the concentration of growth factor at the far end of the tissue is negligible

$$C = 0, \quad x = L. \quad (S4)$$

With this in mind, under the conditions of a constant surface concentration

$$C = C_0, \quad x = 0 \quad (S5)$$

the concentration profile takes the form

$$C / C_0 = \frac{1}{2} e^{-x/\ell} \text{erfc} \left( \frac{x}{2 \sqrt{Dt}} - \sqrt{k t} \right) + \frac{1}{2} e^{x/\ell} \text{erfc} \left( \frac{x}{2 \sqrt{Dt}} + \sqrt{k t} \right). \quad (S6)$$

where

$$\ell = \sqrt{\frac{D}{k}}. \quad (S7)$$
Thus, clearance gives rise to a length scale $\ell$ that is independent of the dimensions of the tissue and is inversely related to the clearance rate constant. Correspondingly, using Danckwert’s method $^2$ it is possible to show that net tissue deposition $M$ depends on time as

$$M = C_0 \ell \text{erf}(\sqrt{kt}).$$  \hspace{1cm} (S8)

Thus, $k^{-1}$ operates as the time scale for the tissue to approach its steady state deposition

$$M = C_0 \ell$$ \hspace{1cm} (S9)

and distribution

$$C = C_0 e^{-x/\ell}.$$ \hspace{1cm} (S10)

Hence, increasing the clearance rate constant results in less drug penetration and more significant localization near the drug source. On the contrary, as the clearance rate constant tends to zero, the limitation on drug penetration is lifted ($\ell \gg L$) such that drug penetration and deposition reduce to the classical linear diffusion limits

$$C / C_0 \approx \text{erfc}\left(\frac{x}{2\sqrt{Dt}}\right)$$ \hspace{1cm} (S11)

and

$$M \approx 2C_0 \sqrt{Dt}/\pi.$$ \hspace{1cm} (S12)

The preceding analysis wherein the surface concentration was held constant provides insights on drug transport during the initial burst release phase. Subsequently drug release from the polymer device occurs at an essentially constant rate. Consider next the extreme limit wherein the flux $F_0$ rather than the concentration is held constant at the surface

$$-D \frac{\partial C}{\partial x} = F_0, \quad x = 0$$ \hspace{1cm} (S13)
The steady state distribution of drug implied by Eq. S1 is then

\[ C = (\ell F_0 / D) e^{-x/\ell}. \]  \( \text{(S14)} \)

Thus, in the face of first order clearance, steady state drug distribution is always exponential with a length scale \( \ell \); device release kinetics is seen to only impact the steady state concentration of drug at the device:tissue interface \( (x=0) \). Integrating the steady state distribution profile Eq. S14 over the entire tissue we find that steady state tissue deposition \( M \) scales linearly with flux and inversely with the apparent clearance rate constant

\[ M = \frac{F_0}{k}. \]  \( \text{(S15)} \)

Using the Danckwert’s method\(^2\), it is possible to derive the time dependent counterpart of Eq S15, as

\[ M = \frac{F_0}{K} (1 - e^{-kt}) , \]  \( \text{(S16)} \)

thus confirming that \( k^{-1} \) also operates as the time scale for the attainment of steady state tissue deposition when drug is released at a constant flux from the polymeric device.

**Analytical Model Calculation of Cumulative FGF Clearance**

Cumulative FGF washout through microvascular clearance, between times \( t_1 \) and \( t_2 \) can be calculated from the experimental value of clearance constant \( k \) and the total deposition \( M \) (Eqn. S8) by the following relationship:

\[ CL = \int_{t_1}^{t_2} kMd\tau = \int_{t_1}^{t_2} kC_0 \ell \text{erf}\left(\sqrt{k\tau}\right) d\tau \]  \( \text{(S17)} \)
Recombinant FGF2 Production

Recombinant FGF2 was expressed in *Escherichia coli* strain FICE-127 transformed by plasmid vector pFC80 that confers resistance to ampicillin and encodes FGF2 under the control of the tryptophan promoter (the transformed FICE-127 strain was a gift from John Heath, University of Birmingham, and was originally constructed by Antonella Isacchi, Amersham Pharmacia Biotech and Upjohn). FICE-127 cells containing pFC80 were inoculated into LB Medium (MP Biomedicals) containing 0.29 mmol/L (10 mg/dL) of ampicillin (Invitrogen) and grown overnight at 37°C in a shaker at 250 RPM. The inoculum was diluted 1:100 in M9 Minimal Medium (Fisher Scientific) containing 1 g/L amino acids (Becton Dickinson) without tryptophan to induce protein production. Cells were grown for 6 h at 37°C in a shaker at 250 RPM. Cells were collected by centrifugation at 8000 RPM for 10 m and kept frozen at -80°C. Frozen cell pellets were resuspended in 6.94×10^{-2} mmol/L (100 mg/dL) lysozyme in GET buffer (100 mmol/L Glucose, 10 mmol/L EDTA, and 50 mmol/L Tris, pH 8.0), vigorously agitated for 5 m and homogenized (Polytron; Kinematica) 5 times for 30 s each with break periods of 60-90 s at 4°C to prevent overheating and denaturation of proteins.

Bacterial lysate was collected by centrifugation at 9,000 RPM for 10 m at 4°C. FGF2 was then purified using affinity chromatography with FPLC (Pharmacia Biotech). Lysate was loaded into 5 mL heparin Sepharose column (HiTrap, GE Healthcare) and allowed to bind for 2 h at 4°C. The FPLC system was programmed to wash the column with phosphate buffered saline (PBS) containing incrementally concentrated NaCl with a linear gradient (150 mmol/L to 2000 mmol/L). Elutions were collected in sequential 1 mL fractions, with FGF2 eluting at 1400 mmol/L to 1600 mmol/L NaCl. The FGF2 solution was desalted by centrifugation using
centrifugal filters with 10 kDa molecular weight cut-off (Centricon, Millipore). After each purification, the presence of FGF2 was confirmed at a 18kDa band using SDS-PAGE, and protein concentration was quantified with a BCA assay (Pierce). Bioactivity of FGF2 was confirmed by in vitro proliferation assays using bovine aortic endothelial cells.

*Fluorescence Labeling of FGF2*

A heparin Sepharose column (HiTrap, GE Healthcare) was loaded with 1 mL of 0.12 mmol/L (200 mg/dL) FGF2 in PBS and allowed to bind for 1 h at room temperature. The FGF2-loaded column was washed with 5 mL of 100 mmol/L NaHCO₃ to increase the pH to 8.3 and loaded with 1 mL of 1.60 mmol/L (100 mg/dL) Texas Red succinimidyl ester (Invitrogen). Texas Red succinimidyl ester was allowed to react with FGF2 for 10 m at room temperature. The column was then placed in-line on the FPLC system, and FGF2 was eluted as described above. Elutions of 1 mL were collected and assayed for protein content (absorbance at 280 nm, FPLC System) and Texas Red fluorescence intensity (595 nm/615 nm excitation/emission wavelengths, Fluoroskan II, Lab Systems Oy). Texas Red was appropriately conjugated to FGF2 as indicated by the concurrence of the elution peaks of fluorescence intensity and protein concentration. Both were came off the column between 1400 to 1600 mmol/L NaCl, similar to the elution range of unlabeled FGF2, suggesting that Texas Red conjugation did not change the heparin binding properties of FGF2. Buffer exchange was performed on the Texas Red labeled-FGF2 (TR-FGF2) solution by centrifugation as above. SDS-PAGE indicated that the molecular weight of TR-FGF2 is not significantly different from that of FGF2. Fluorescence intensity was calibrated to protein concentration prior to delivery using microplate fluorometry (Fluoroskan).
Recombinant S35-FGF1 Production

$^{35}$S-FGF1 was chosen as a model for FGF in the *in-vivo* setting because its high detection sensitivity allows for the possibility of tracking spatial drug distribution in the setting of controlled delivery of therapeutic doses. It also presents a safer alternative to using $^{125}$I labeled FGF2 *in-vivo*. Recombinant human FGF1 was expressed in *Escherichia coli* strain BL21-pLysS transformed by plasmid, pET3c that confers resistance to ampicillin and encodes FGF1 (obtained as a gift from the late Dr. Thomas Maciag, Maine Medical Center, Portland, ME). Transfected bacteria stocks were added to LB medium (MP Biomedicals) containing 100 µg/mL Carbenicillin and 35 µg/mL Chloramphenicol (Sigma-Aldrich) and incubated with vigorous shaking at 250 RPM at 37°C overnight. Bacteria culture was then diluted in antibiotics-free LB medium (1:40 v/v of bacteria/medium) and incubated with vigorous shaking at 250 RPM and the optical density of the solution was measured at 600 nm. When cell culture reached an optical density of 0.6-0.8 bacteria were centrifuged and resuspended in DMEM medium deficient in L-cysteine and L-methionine (Invitrogen) supplemented with 1 % L-glutamine and 7.15 mCi of $^{35}$S (Promix L-$^{35}$S]Methionine and Cysteine, GE Healthcare Life Sciences) and buffered with 25 mM HEPES (Invitrogen) and then induced with 0.4 mM IPTG for 3 h with shaking at 250 RPM at 37°C. Cells were then collected by centrifugation at 8000 RPM for 10 m and kept frozen at -80°C.

Frozen cell pellets were resuspended in 1mg/ml lysozyme in GET buffer (50 mM Tris, 10 mM EDTA, 100 mM glucose, pH 8.0), mixed well for 5 m and then homogenized (Polytron; Kinematica) 5 times for 30 s each with break periods of 60-90 s at 4°C to prevent overheating and denaturation of proteins. Cell lysate was collected by centrifugation at 9,000 RPM for 10 m
at 4°C. ³⁵S-FGF1 was then purified using affinity chromatography with FPLC. Bacterial lysates were loaded into 5 ml heparin sepharose column (HiTrap Heparin HP column, GE Healthcare Life Sciences) and allowed to bind for 1 h at room temperature. The FPLC system was programmed to wash the column with PBS and gradually increase NaCl concentration with a linear gradient (0.15-2 M). Elutions of 1 mL were collected and assayed for protein concentration (absorbance at 280 nm, FPLC System) and radioactivity (2500TR Liquid Scintillation Analyzer, Packard). Elution peaks of protein concentration and radioactivity coincided. The ³⁵S protein product came out of the heparin sepharose column at 1.4 M to 1.6 M NaCl, similar to FGF1, and indicating ³⁵S was incorporated in FGF1. ³⁵S-FGF1 solution was desalted by centrifugation using centrifugal filter devices with a 10 kDa molecular weight cut-off (Centricon, Millipore). After each purification, the presence of FGF1 was confirmed at a 18 kDa band using SDS-PAGE and protein quantification was carried out using BCA assay (Pierce). Bioactivity of FGF1 was confirmed in proliferation assays of bovine aortic endothelial cells. FGF1 was further purified from any endotoxin contamination using endotoxin removing columns (Detoxi-Gel AffinitiPak columns, Pierce), and the FGF1 purity was confirmed with limulus amebocyte lysate assay (Pyrotell-T, Associates of Cape Cod Inc., MA). Purification was continued (typically 2-3 times) until endotoxin concentration is below 0.01 EU/µg FGF1.

**Fabrication and Kinetics of Controlled Release Device**

A slurry mixture of heparin-Sepharose beads/alginate solution was prepared by mixing sterilized heparin-Sepharose microbeads (GE Healthcare) with filter-sterilized sodium alginate (Sigma-Aldrich, 5 %), placed in a customized 20mm × 20mm × 1mm glass mold, and incubated overnight in filter-sterilized 10 % CaCl₂. The heparin-Sepharose embedded sodium alginate
material is exposed to CaCl₂ through two open surfaces of the mold. The gel was further incubated in the 10 % CaCl₂ for 24 h to allow thorough cross-linking of the polymer after being taken out of the mold under UV light for sterilization. The hardened polymeric gel device was then incubated in ³⁵S-FGF1 solution for 48 h prior to experiment to allow complete and uniform loading of drug.

To characterize ³⁵S-FGF1 release kinetics from the device, 8 mm diameter circular shape devices were made from the 20 × 20 × 1 mm³ polymeric slab using 8 mm biopsy punch (Miltex). These circular devices were incubated in 1 ml PBS and gently agitated throughout the experiment with a shaker. At various time points the elution mixture was collected and assayed for ³⁵S activity using liquid scintillation counter (Packard). Fresh PBS was used to renew the elution buffer.
Supplemental Results

*FGF is Washed-out Through Microvascular Clearance Followed Ex-vivo Myocardial Delivery*

$^{35}$S-FGF1 was observed in the perfusate at the outflow soon after $^{35}$S-FGF1 was delivered at epicardial surface (Figure S2), suggesting that capillary washout was indeed responsible for the limited penetration of this growth factor in the presence of perfusion (Figure 1). These results were further compared to analytical model results of cumulative drug clearance (Eqn S17) calculated using pharmacokinetic parameters of FGF-2 ($k = 1.15 \pm 0.06 \times 10^{-4}$ s$^{-1}$ and $D=0.02 \mu$m$^2$/s) derived from experiment. The experimental results fit the model well within one order of magnitude, suggesting that the pharmacokinetics of $^{35}$S-FGF1 are well explained by our diffusion with clearance model and moreover that the clearance constants and diffusivities of $^{35}$S-FGF1 and TR-FGF2 are similar.
Figure Legends:

FIGURE S1: Isolated perfused heart apparatus. Rat coronary arteries were perfused antegrade through an aortic canula at constant physiologic mean pressure while a constant, well mixed drug source was applied to the epicardial surface. Spatial drug distribution was quantified in myocardial tissue regions exposed to drug. High magnification schematic illustrates the examined physiologic forces: drug diffusion within tissue and clearance through convection by intravascular flow after permeation across capillary wall.

FIGURE S2: FGF is Washed-out Through Microvascular Clearance Followed Ex-vivo Myocardial Delivery. $^{35}$S-FGF1 in the outflow perfusate was measured as a function of time after local epicardial $^{35}$S-FGF1 delivery (n=3). Experimental washout of $^{35}$S-FGF1 (in blue) is well explained by Eq. S17 (magenta line) using the parameter values of TR-FGF2.
References


FIG-S2

Cumulative $^{35}$S-FGF1 Outflow vs. Time (hr)

- Model
- Expt