

Resonance Energy Transfer for Assessing the Molecular Integrity of Proteins for Local Delivery

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Abstract: It remains unclear whether the limitations to the therapeutic potential of angiogenic growth factors stem from pharmacokinetic concerns related to inadequate delivery or from a reduced sensitivity of target tissues. Here, we report a novel method using resonance energy transfer to assess the molecular integrity of proteins after local delivery. As an example, we labeled fibroblast growth factor-2 with a fluorescent donor and nonfluorescent acceptor pair, tetramethylrhodamine and QSY-7, and demonstrate in an ex vivo bovine carotid artery model that this growth factor is not limited by proteolytic constraints imposed by the tissue. Our data indicate that FGF-2 is unlikely to be degraded within the arterial wall and suggest that pharmacokinetic limitations alone cannot fully explain the muted response seen thus far in therapeutic angiogenesis. In general, resonance energy transfer may serve as a novel approach to assess the molecular integrity of protein-based therapies in local delivery. © 2004 Wiley Periodicals, Inc.

Keywords: FGF.2; therapeutic angiogenesis; local delivery; resonance energy transfer; Pharmacokinetics

INTRODUCTION

Effective delivery of growth factors and therapeutic proteins in general remains a challenge (Jain, 1998). Issues of targeting, sustained presentation, and complications of pharmacokinetics and pharmacodynamics within tissues are continued limitations to robust biological responsiveness (Jain, 1998). Physiological transport forces, including convective and diffusive sinks, can restrict effective drug deposition and targeting (Hwang et al., 2001; Mahoney and Saltzman, 1999). Moreover, cellular receptor-ligand trafficking and both specific and nonspecific enzymes that mediate proteolytic degradation may further dictate their fate (Chu et al., 1996; Rippley and Stokes, 1995). Due to the plethora of

mechanisms that can modulate growth factor function, the ability to define the effective delivery of these proteins and, more importantly, determine whether they are molecularly intact is critical in optimizing their therapeutic potential.

Traditional approaches to follow the delivery of therapeutics through tissues predominantly include tracking fluorescent and radioactive labels conjugated to a therapeutic entity. Neither, however, readily provides information about the molecular integrity of the protein in the complex environment into which they are delivered. Typically, tracking of label is presumed to correlate with the location of the protein. However, this assumption may be less valid for proteins with short penetration distances in tissues, extensive metabolism by nonspecific and specific pathways, dramatic differences in size of intact versus degraded proteins and rapid dispersion of low molecular weight peptide fragments. Moreover, since typically injured tissues are the targets of these therapies, the local environment is likely to be rich with enzymes requisite for remodeling matrix and mediating inflammation and the response to injury. Growth factors and other protein therapeutics may potentially be inhibited from biological action due to substantial degradation, yet traditional approaches cannot readily discern this mechanism of failure from inherent biological or biochemical unresponsiveness. Indeed, these pharmacological issues are perhaps most profound in the field of therapeutic angiogenesis in which growth factors are delivered to ischemic myocardium in hopes of encouraging neovessel development (Harada et al., 1994; Kornowski et al., 2000; Laham et al. 1999a, 1999b; Sellke et al. 1998a, 1998b).

Resonance energy transfer (RET) is the non-radiative transfer of energy between appropriately oriented dipoles of two fluorophores, located within 10–100 Å (Clegg, 1995; Wu and Brand, 1994). The inherent nature of this dipole interaction, its $1/R^6$ spatial dependence, allows it to serve as a molecular ruler, delineating distances between the two groups at the molecular scale. As such, RET can provide information about the molecular integrity of a protein

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(Bastiaens et al., 1996; Ng et al., 1999; Uchiyama et al., 1996; Yaron et al., 1979). In this report, we demonstrate the application of RET to the study of local growth factor pharmacology and show the potential of this approach to provide molecular information pertinent for local delivery.

EXPERIMENTAL PROCEDURES

Synthesis of Quenched FGF-2

Fibroblast growth factor, FGF-2, a gift from Dr. A. Protter, was loaded onto a 1 ml HiTrap heparin column at 10 $\mu\text{g}/\mu\text{L}$ (Amersham Pharmacia-Biotechnology, Piscataway, NJ), preloaded with 1 mg/mL ovalbumin in PBS, pH 7.0, to minimize nonspecific binding. Growth factor was allowed to bind for at least 1 h on ice with or without ^{125}I -Bolton-Hunter-Reagent-FGF-2 in some cases to enhance detection. A 20-fold molar excess of QSY-7 maleimide dye (Molecular Probes, Eugene, OR) was to the column in 1 mL of 1 mg/mL ovalbumin in PBS, pH 7.0. The reaction was allowed to proceed for at least 7 h on ice. Next, using fast protein liquid affinity chromatography (FPLC) (Amersham Pharmacia Biotechnology), the unconjugated dye was removed with at least 200 column volumes of 0.15 M NaCl in PBS, pH 7.4. The elution was monitored by γ -counting and measuring absorbance at 280 nm. In addition, the eluent was visually assessed for the presence of the non-fluorescent dye. The pH was then changed to pH 8.5 with sodium bicarbonate. A 20-fold molar excess of TAMRA-TMR succinimidyl ester (Molecular Probes) was diluted into 1 mL of sodium bicarbonate buffer, pH 8.4, centrifuged to remove precipitates, and loaded onto the HiTrap column. The reaction was allowed to proceed overnight on ice. The column was then again placed in-line on the FPLC, and the unconjugated TAMRA-TMR-SE dye was eluted with approximately 200 column volumes of 0.15 M NaCl in PBS, pH 7.4. Elution was monitored by γ -counting, measuring absorbance at 280 nm, and fluorescence using Fluoroskan II, excitation 544 and emission 590 nm (Lab Systems Oy, Helsinki, Finland). The FGF-2 dual-labeled conjugate was subsequently eluted off using a step gradient from 0.15 to 2 M NaCl in 10 mM phosphate buffer, pH 7.4. Fractions were collected and subjected to centrifugation to desalt and remove unbound, unconjugated dye using a membrane with a nominal molecular weight cutoff of 10,000 g/mol (Millipore, Bedford, MA). Dye/FGF-2 labeling ratios were calculated as described in product literature, and protein concentrations were determined by absorbance at 280 nm or by ELISA (R&D Products, Minneapolis, MN).

Proteolytic Assays

To define the functional response of the quenched FGF-2 conjugate to protease digestion, conjugate was mixed with PBS²⁺, chondroitin sulfate A, or heparin (final concentration 100 $\mu\text{g}/\text{mL}$) and allowed to pre-equilibrate for at least 10 min on ice. Next, trypsin (50 mg/mL), plasmin (1 unit/mL), or

thrombin (50 units/mL) were mixed into 100 mL of PBS²⁺. The proteolytic solutions mixed with the quenched conjugate were subsequently added to 96-well plates and assayed for fluorescence activity as a function of time at room temperature using a 96-well plate Fluoroskan II fluorescent reader (Lab Systems Oy). Results were normalized to the initial fluorescence at time zero, $t = 0$, and plotted as the mean \pm standard deviation of at least 3 independent replicates.

Ex Vivo Tissue Model

To assess the proteolytic activity of quenched FGF-2 in the arterial wall, bovine carotid arteries were harvested as previously described (Hwang et al., 2001; Hwang and Edelman, 2002). Next, the arteries were sectioned into 5-mm cylindrical segments and incubated in 2% penicillin-streptomycin in PBS²⁺ at 4°C to minimize bacterial contamination. Tissues were then cultured routinely in Dulbecco's Modified Eagle's Medium and 10% fetal bovine serum under 10% CO₂ at 37°C. To assess the health of the tissue during ex vivo culturing, the LIVE/DEAD assay by Molecular Probes (Eugene, OR) was used at various times from 1 to 8 days.

Quenched FGF-2 substrates were incubated in DMEM/10% FBS at concentrations well exceeding the binding affinity constant, K_d , to mimic the concentrations used in in vivo studies. The solutions were then added to the explanted carotid segments alone, in the presence of heparin (final concentration 100 $\mu\text{g}/\text{mL}$, Pharmacia & Upjohn, Peapack, NJ), or in the presence of protease inhibitors (Mini-Complete, Roche Molecular Biochemicals, Indianapolis, IN). Quantitative fluorescent images were taken 48 h after initial incubation of quenched FGF-2 to ensure adequate growth factor penetration (Dowd et al., 1999). For imaging, cylindrical carotid segments were cryosectioned into 20- μm slices (Cryotome SME, Shandon) and imaged immediately using a fluorescence microscope (Optiphot-2, Nikon) with a 465–495 nm excitation filter, a 505 nm dichroic filter, and a 515–555 nm barrier filter (Hwang et al., 2001; Hwang and Edelman, 2002). Specific regions of interest within the arterial media were highlighted and quantified for mean intensity, using approximately 15,000–20,000 pixels after conversion of the color images to intensity plots using software from IP Lab Spectrum. Equilibrium control experiments defined the functional response of the fluorescent microscope to varying concentrations of free tetramethylrhodamine incorporated into carotid arteries (Wan et al., 1999). Images shown are representative sections randomly sampled at ten locations throughout the length of the explant.

RESULTS

Fibroblast growth factor-2 (FGF-2), a potent angiogenic molecule involved in the development of vessels, is released upon endothelial damage to mediate wound healing. Because of its critical role in also mediating the development

of angiogenic vessels, it has been investigated as a potential candidate for therapeutic angiogenesis. Consequently, we chose this molecule to see if insight into its local pharmacology might explain its muted effects observed thus far in clinical trials.

Conjugation

FGF-2 was labeled using standard protein chemistry methods with the fluorescent donor tetramethylrhodamine succinimidyl ester (TMR) and the nonfluorescent acceptor QSY-7 maleimide, which together form a RET pair. Dual conjugation of FGF-2 with this RET pair was achieved sequentially on a heparin affinity chromatography column (Healy and Herman, 1992). After extensive washing to remove unlabeled dye, the RET conjugate was scanned for absorbance. Two peaks centered at 534 nm and 562 nm corresponding to the peaks of TMR and QSY-7, respectively, were observed in the desalted 2 M NaCl eluent, suggesting dual labeling (Fig. 1A). In contrast, no peaks were observed in the absorbance scans of PBS²⁺ and the ultrafiltrate obtained after centrifugation. Using molar absorptivities provided by the manufacturer, the dye/FGF-2 labeling ratios were calculated using the equation

$$Abs_{total\ at\ dye_{1,2}} = Abs_{1,2} + Abs_{2,1} \times CF_{1,2},$$

where $Abs_{1,2}$ is the absorbance of dye at the appropriate wavelength and $CF_{1,2}$ is the correction factor for dye_{1,2} at the

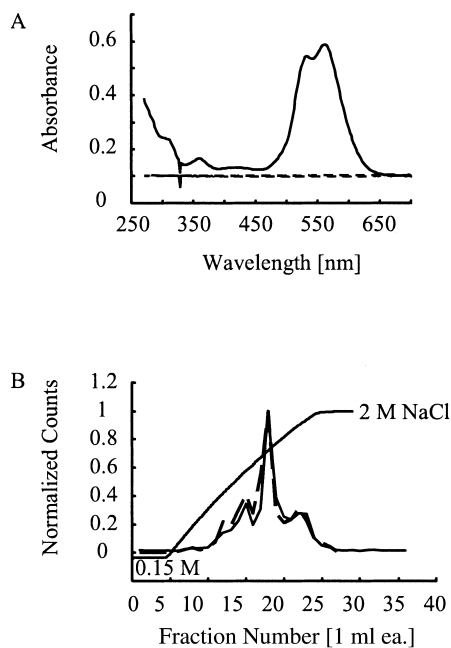


Figure 1. Synthesis of the quenched conjugate. (A) Absorbance of desalted conjugate (solid line) versus PBS²⁺ and ultrafiltrate through 10,000 MWCO filters (dotted lines). Unlabeled FGF-2 has a similar absorbance profile as the PBS²⁺ and ultrafiltrate curves in the range from 400 to 700 nm (data not shown). (B) Elution of conjugate with NaCl gradient from HiTrap heparin-Sepharose column to determine binding activity. Elution of conjugate (solid line) versus non-fluorescently labeled species (dashed line).

maximum absorption wavelength of dye_{2,1}. Averaged over multiple labeling experiments, FGF-2 was labeled at a molar ratio of 0.91 ± 0.08 for TMR and 0.96 ± 0.05 for QSY-7 with respect to FGF-2.

To characterize the biological function of this RET conjugate, we determined whether dual RET labeling of FGF-2 negatively impacted on its heparin binding activity, a well-recognized property important for the pharmacokinetic targeting of this molecule and subsequent biological effect. Quenched fluorescent conjugate that was loaded onto a 1-mL HiTrap heparin column was probed for binding specificity using a salt gradient after extensive washing with >20 column volumes. This resulted in a maximal peak centered around 1.5 M NaCl and multiple smaller peaks consistent with heterogenous lysine radiolabeling (Fig. 1B). Comparison of the elution profile of the fluorescently quenched conjugate with that obtained using the radiolabeled control showed that dual RET labeling did not appreciably impact upon its heparin binding activity (Fig. 1B). Concomitant proliferation studies using NR6 Swiss 3T3 fibroblasts demonstrated that there was no difference in cell number as compared to unlabeled growth factor after dosing for 3 days at saturating concentrations (data not shown).

Proteolytic Sensitivity of FGF-2 Conjugate

To quantify the response to proteolysis of quenched FGF-2, the conjugate was incubated with and without heparin or trypsin, and fluorescence was sampled 24 h later (Fig. 2A). As shown, there was a (12.40 ± 1.67) -fold relative increase in fluorescence observed in samples not protected by heparin but subjected to trypsin digestion compared to control samples without heparin and trypsin, 1.00 ± 0.24 . Notably, there was a smaller (2.14 ± 0.39) -fold increase in fluorescence seen in samples that included heparin, suggesting escape from protection that is consistent with the reversible nature of binding interactions between FGF-2 and heparin.

This protective benefit afforded by heparin was specific as incubation with chondroitin sulfate did not protect the conjugate from trypsin digestion [(4.32 ± 0.28) -fold increase with chondroitin sulfate versus (1.58 ± 0.1) -fold increase with heparin] (Fig. 2B). To confirm that this effect was due to heparin binding and thus should be reversible with ionic disruption and not chondroitin sulfate interference with trypsin activity, conjugates were pre-incubated with heparin to allow for initial binding. After 10 minutes, trypsin was added to the system. Next, either a high concentration of salt or PBS²⁺ was added to quenched FGF-2. As shown in Figure 2C, the addition of salt relieved the protection afforded by heparin resulting in a dramatic increase in fluorescence. In contrast, PBS²⁺ had no effect. Taken together, these results confirm that FGF-2 can be successfully labeled with a RET dye pair without disrupting its essential heparin binding properties.

To further define the proteolytic sensitivity of the quenched conjugate, the proteases plasmin and thrombin were added to the quenched growth factor separately. Relative

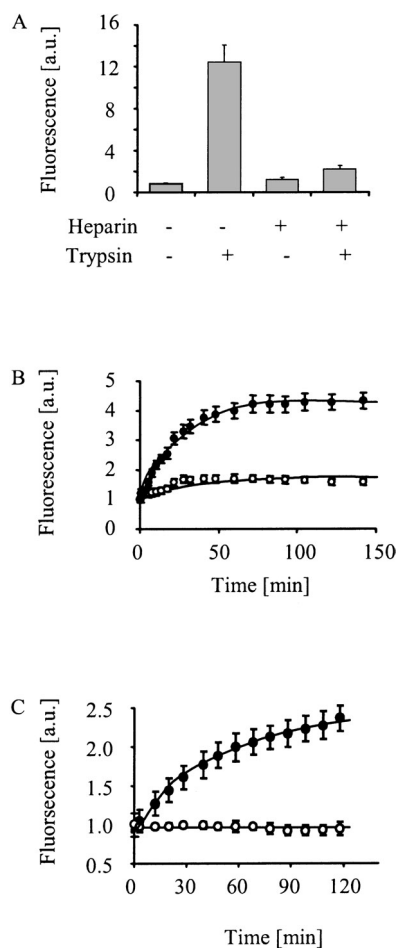


Figure 2. Characterization of quenched conjugate. (A) Exhaustive digestion of quenched conjugate in the presence or absence of heparin (100 $\mu\text{g}/\text{mL}$) and/or trypsin (10 $\mu\text{g}/\text{mL}$) at 24 h at 37°C. (B) Effect of chondroitin sulfate A (●) or heparin (○) at 100 $\mu\text{g}/\text{mL}$ on protection of quenched conjugate from digestion by trypsin. (C) Effect of 2 M NaCl (●) or PBS (○) on disruption of protection by heparin of quenched conjugate from trypsin digestion.

fluorescence increased (5.25 ± 0.14)-fold upon the addition of plasmin versus samples concurrently protected by heparin, (0.89 ± 0.10)-fold (Fig. 3A). In contrast, no increase in fluorescence was detected upon the addition of PBS²⁺ or thrombin after 1 h (Fig. 3B) or even at 24 h (data not shown). Together, these data are consistent with published reports demonstrating that FGF-2 is sensitive to plasmin, but not thrombin, digestion and confirm the ability of this quenched conjugate to provide accurate information about the proteolytic sensitivity of FGF-2 (Lobb, 1988; Rosengart et al., 1988; Saksela et al., 1988).

Tissue Pharmacokinetics

To examine the tissue pharmacology of this conjugate, we used an explanted, tissue culture arterial model (Koo and Gotlieb, 1989, 1992), to simulate the end delivery of angiogenic growth factors to distal vessels in the

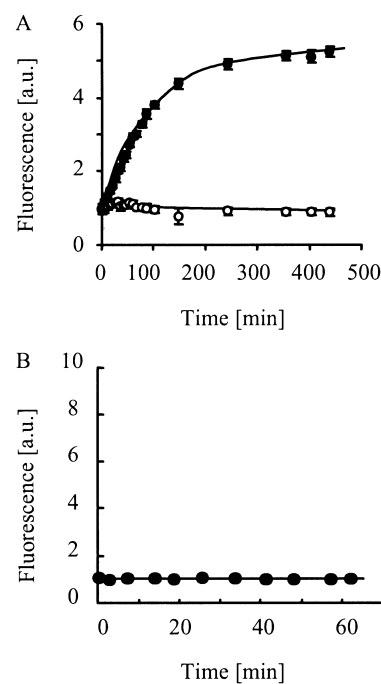


Figure 3. Proteolytic sensitivity of conjugate. (A) Effect of PBS (●) or heparin (○) on protecting quenched conjugate from digestion by plasmin. (B) Effect of thrombin incubation with quenched FGF-2.

myocardium. Clinically, this might represent the delivery achieved for instance through intra-arterial deposition by catheter delivery or through the controlled-release from polymeric devices lodged distally in coronary vessels. Importantly, the use of this non-perfused explant model reasonably mitigates the substantial loss of growth factor due to systemic clearance that is known to occur in vivo, which is not the subject of this report, and therefore allowed us to focus specifically on the proteolytic constraints of tissue pharmacology in local growth factor delivery in a readily accessible manner.

Control studies demonstrated that these explants remain viable for at least 8 days as assessed by the Molecular Probes' LIVE/DEAD assay. Moreover, over this time period, there was minimal change in the number of cells that stained punctate red, indicative of cell membrane compromise ($79.5\% \pm 3.8\%$ on Day 1 and $77.5\% \pm 8.6\%$ on Day 8). Of note, transport experiments were conducted over 2 days, as full penetration of the arterial explant by the RET conjugate is achieved within this time frame.

To assess the potential for proteolytic degradation by arterial tissue, quenched FGF-2 was incubated alone as the experimental, with protease inhibitors, or with heparin in 10% FBS/DMEM. As a positive control, quenched FGF-2 was pre-treated with trypsin for 4 h at 37°C before incubation. Explants were then assessed for fluorescence at 48 h at 37°C, as an order-of-magnitude analysis for diffusion suggests full penetration of the conjugate within this time (Dowd et al., 1999). Representative images of explants are shown after axial sampling at 20- μm increments (Fig. 4A,B).

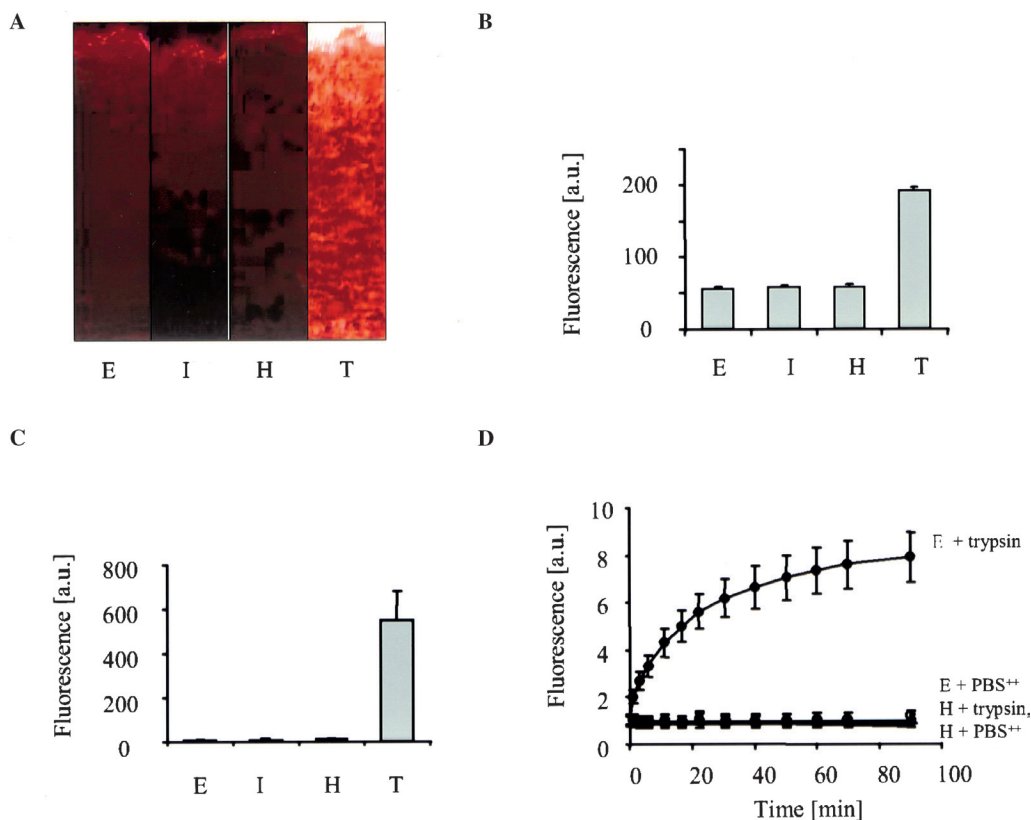


Figure 4. Tissue pharmacology of quenched FGF-2. (A) Representative images of fluorescence within explanted carotids subjected to experimental quenched FGF-2 alone, or with protease inhibitors, heparin or trypsin pretreatment, as described in Methods. (B) Quantitative fluorescence intensity within the arterial media. (C) Fluorescence of corresponding conditioned media. (D) Treatment with trypsin or with PBS²⁺ of conditioned medias of experimental quenched FGF-2 alone or quenched FGF-2 + heparin, as denoted. (Abbreviations: E = experimental conjugate alone, I = with protease inhibitor, H = with heparin, T = trypsin pre-treated conjugate).

In all specimens, no fluorescence was observed in explants incubated with quenched FGF-2 alone, with quenched FGF-2 and a composite of protease inhibitors, and with quenched FGF-2 and heparin together, suggesting minimal proteolytic degradation of FGF-2 by the vascular wall (Fig. 4A,B). In contrast, in explants incubated with trypsin predigested conjugate, fluorescence was readily observed in the arterial media, suggesting sufficient signal within this system to permit detection of degradation if pharmacologically significant.

To confirm these results, conditioned media samples were quantified for fluorescence using a 96-well plate reader. In samples incubated with quenched FGF-2 alone, quenched FGF-2 and protease inhibitors, and quenched FGF-2 and heparin, unappreciable fluorescence was detected (Fig. 4C). In contrast, significant fluorescence was measured in the conditioned media obtained from carotid samples incubated with trypsin predigested quenched conjugate (Fig. 4C). Moreover, further experiments confirmed that quenched FGF-2 was not inactivated as an artifact in the samples that demonstrated no fluorescence, as there was a dramatic increase in fluorescence upon the post-experimental addition of trypsin to these conditioned media samples (Fig. 4D). In contrast, quenched FGF-2 incubated with heparin when

treated with either PBS²⁺ or trypsin demonstrated no increase in fluorescence, confirming again that heparin protects this conjugate from degradation herein (Fig. 4D). Taken together, quenched FGF-2 is minimally degraded within this bovine explant model over a time course adequate for full penetration.

DISCUSSION

In this work, we offer resonance energy transfer as a means by which to examine the physical integrity of locally delivered protein therapeutics. Fibroblast growth factor is a potent vascular cell mitogen and angiogenic factor, whose biologic effects are intimately linked to its mode of delivery (Dinbergs et al., 1996). The fragility of the compound and its need for heparin-binding to form a trimolecular signaling complex make it particularly challenging to label and inherently difficult to deliver (Moscatelli, 1992; Nugent and Edelman, 1992). In these studies, quenched FGF-2 demonstrated unappreciable degradation in an ex vivo carotid model of therapeutic angiogenesis. While trypsin predigested conjugate was sufficiently detectable within the arterial wall and conditioned media and thus confirmed

ample sensitivity within this system, our data suggest minimal degradation of quenched FGF-2 within the arterial wall over a time course relevant for adequate penetration (Fig. 4). These results are consistent with the conclusion that despite inherent sensitivity to protease degradation as characterized in Figure 2, FGF-2 is not likely to be substantially constrained by this process in local delivery.

Saltzman and Radomsky (1991) first postulated that extensive proteolytic degradation is a significant concern for the effective local delivery of proteins. They noted that while spatially averaged drug concentrations approach maximal concentrations when degradation is less dominant than diffusion, significant concentration gradients exist when diffusion is overshadowed by degradation. Indeed, several groups have confirmed this prediction experimentally and computationally (Chu, 1998; Mahoney and Saltzman, 1999; Rippley and Stokes, 1995). These issues are perhaps more profound considering the environment into which many local growth factor or protein therapies are targeted. For instance, tissue ischemia, injury, and necrosis may result in the indiscriminant release of normally sequestered enzymes that can rapidly degrade proteins in a nonspecific manner. In addition, local changes in pH may inherently denature growth factors or alter their ability to interact avidly with cell surface receptors. Taken together, tissue pharmacokinetics may dramatically alter the molecular integrity of delivered therapeutics.

That quenched FGF-2 remained intact in the arterial wall in this system could be due to a variety of reasons. For instance, there could be reduced presence or activity of enzymes capable of degrading FGF-2, such as trypsin- or plasmin-like enzymes. This seems less likely, since the explant arterial wall model used in this study has been shown to serve as an *in vitro* model of intimal hyperplasia, a state of injury involving remodeling by matrix proteases, cell migration, and proliferation (Koo and Gotlieb, 1991, 1992). Alternatively, the quenched conjugate may have been partially degraded, yet because of a poor signal to noise ratio, the fluorescence was undetectable. While this might be a possibility, it is nevertheless irrelevant for local growth factor delivery in which growth factor concentrations that far exceed the equilibrium receptor binding constant are typically employed, and which we simulated here. Labeling of FGF2 on lysines and cysteines might prevent putative enzymes in the arterial wall from recognizing key domains for interaction. However, given the low one-to-one molar labeling ratios of dye to growth factor used in these studies, this is a less significant concern. Most likely, heparan sulfate proteoglycans found within extracellular matrix and on cellular surfaces stabilize FGF-2 and protect it from degradation and denaturation (Sperinde and Nugent, 2000; Vlodaysky et al., 1991; Vlodaysky et al., 1987). Indeed, several reports have demonstrated the role of heparan sulfate proteoglycans in protecting growth factor as a kinetic storage depot for later presentation (Flaumenhaft et al., 1990; Rifkin et al., 1990, 1991; Saksela et al., 1988). Stabilization of FGF-2 through heparin binding to enhance its function would make sense

especially as FGF-2 lacks a signal sequence and is released upon endothelial damage (McNeil et al., 1989).

In general, failure of local growth factor therapy can be due to only three reasons: (i) inadequate targeting and delivery to the tissue site, (ii) adequate targeting and delivery, but in an inadequate molecular state, either denatured or degraded, or (iii) pharmacodynamic differences in tissue responsiveness that mute the response as compared to *in vitro* expectations. In this report, we offer resonance energy transfer as a novel approach to assess the molecular integrity of protein-based therapies in local delivery. Using this approach, we found insignificant proteolysis of FGF-2 and conclude that local delivery of FGF-2 is likely to be minimally constrained by tissue pharmacology in vascular tissues and myocardium, which both contain high amounts of heparan sulfate proteoglycans. Thus, failure of FGF-2 therapy must be attributed to alternative explanations. Of note, two recent studies have suggested that growth factor mediated angiogenesis may require a battery of growth factors given in their physiologically appropriate sequence and timing (Cao et al., 2003; Richardson et al., 2001). Future studies elucidating the complex pharmacokinetics of these growth factors in combination should provide further insight on enhancing the potency of these agents in local delivery.

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References

- Bastiaens PI, Majoul IV, Verveer PJ, Soling HD, Jovin TM. 1996. Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. *EMBO J* 15(16):4246–4253.
- Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y. 2003. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med* 9(5):604–613.
- Chu L. 1998. Ligand transport through cellular matrices and the role of receptor-mediated trafficking. Cambridge: Massachusetts Institute of Technology.
- Chu L, Wiley HS, Lauffenburger DA. 1996. Endocytic relay as a potential means for enhancing ligand transport through cellular tissue matrices: analysis and possible implications for drug delivery. *Tissue Eng* 2(1): 17–38.
- Clegg RM. 1995. Fluorescence resonance energy transfer. *Curr Opin Biotechnol* 6(1):103–110.
- Dinbergs ID, Brown L, Edelman ER. 1996. Cellular response to transforming growth factor- β 1 and basic fibroblast growth factor depends on release kinetics and extracellular matrix interactions. *J Biol Chem* 271(47):29822–29829.
- Dowd CJ, Cooney CL, Nugent MA. 1999. Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding. *J Biol Chem* 274(8):5236–5244.
- Flaumenhaft R, Moscatelli D, Rifkin DB. 1990. Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor. *J Cell Biol* 111(4):1651–1659.

- Harada K, Grossman W, Friedman M, Edelman ER, Prasad PV, Keighley CS, Manning WJ, Sellke FW, Simons M. 1994. Basic fibroblast growth factor improves myocardial function in chronically ischemic porcine hearts. *J Clin Invest* 94(2):623–630.
- Healy AM, Herman IM. 1992. Preparation of fluorescent basic fibroblast growth factor: localization in living retinal microvascular endothelial cells. *Exp Eye Res* 55(5):663–669.
- Hwang CW, Edelman ER. 2002. Arterial ultrastructure influences transport of locally delivered drugs. *Circulation Res* 90(7):826–832.
- Hwang CW, Wu D, Edelman ER. 2001. Physiological transport forces govern drug distribution for stent-based delivery. *Circulation* 104(5):600–605.
- Jain RK. 1998. The next frontier of molecular medicine: delivery of the reapeutics. *Nat Med* 4(6):655–657.
- Koo EW, Gotlieb AI. 1989. Endothelial stimulation of intimal cell proliferation in a porcine aortic organ culture. *Am J Pathol* 134(3):497–503.
- Koo EW, Gotlieb AI. 1991. Neointimal formation in the porcine aortic organ culture. I. Cellular dynamics over 1 month. *Lab Invest* 64(6):743–753.
- Koo EW, Gotlieb AI. 1992. The use of organ cultures to study vessel wall pathobiology. *Scanning Microsc* 6(3):827–834; discussion 835.
- Kornowski R, Fuchs S, Leon MB, Epstein SE. 2000. Delivery strategies to achieve therapeutic myocardial angiogenesis. *Circulation* 101(4):454–458.
- Laham RJ, Hung D, Simons M. 1999a. Therapeutic myocardial angiogenesis using percutaneous intrapericardial drug delivery. *Clin Cardiol* 22(1 Suppl 1):16–19.
- Laham RJ, Rezaee M, Post M, Sellke FW, Braeckman RA, Hung D, Simons M. 1999b. Intracoronary and intravenous administration of basic fibroblast growth factor: myocardial and tissue distribution. *Drug Metab Dispos* 27(7):821–826.
- Lobb RR. 1988. Thrombin inactivates acidic fibroblast growth factor but not basic fibroblast growth factor. *Biochemistry* 27(7):2572–2578.
- Mahoney MJ, Saltzman WM. 1999. Millimeter-scale positioning of a nerve-growth-factor source and biological activity in the brain. *Proc Natl Acad Sci USA* 96(8):4536–4539.
- McNeil PL, Muthukrishnan L, Warder E, D'Amore PA. 1989. Growth factors are released by mechanically wounded endothelial cells. *J Cell Biol* 109(2):811–822.
- Moscatelli D. 1992. Basic fibroblast growth factor (bFGF) dissociates rapidly from heparan sulfates but slowly from receptors. Implications for mechanisms of bFGF release from pericellular matrix. *J Biol Chem* 267(36):25803–25809.
- Ng T, Squire A, Hansra G, Bornancin F, Prevostel C, Hanby A, Harris W, Barnes D, Schmidt S, Mellor H, et al. 1999. Imaging protein kinase C α activation in cells. *Science* 283(5410):2085–2089.
- Nugent MA, Edelman ER. 1992. Kinetics of basic fibroblast growth factor binding to its receptor and heparan sulfate proteoglycan: a mechanism for cooperativity. *Biochemistry* 31(37):8876–8883.
- Richardson TP, Peters MC, Ennett AB, Mooney DJ. 2001. Polymeric system for dual growth factor delivery. *Nat Biotechnol* 19(11):1029–1034.
- Rifkin DB, Moscatelli D, Bizik J, Quarto N, Blei F, Dennis P, Flaumenhaft R, Mignatti P. 1990. Growth factor control of extracellular proteolysis. *Cell Differentiation Dev* 32(3):313–318.
- Rifkin DB, Moscatelli D, Flaumenhaft R, Sato Y, Saksela O, Tsuboi R. 1991. Mechanisms controlling the extracellular activity of basic fibroblast growth factor and transforming growth factor. *Ann NY Acad Sci* 614:250–258.
- Ripley RK, Stokes CL. 1995. Effects of cellular pharmacology on drug distribution in tissues. *Biophys J* 69(3):825–839.
- Rosengart TK, Johnson WV, Friesel R, Clark R, Maciag T. 1988. Heparin protects heparin-binding growth factor-I from proteolytic inactivation in vitro. *Biochem Biophys Res Commun* 152(1):432–440.
- Saksela O, Moscatelli D, Sommer A, Rifkin DB. 1988. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J Cell Biol* 107(2):743–751.
- Saltzman WM, Radomsky ML. 1991. Drugs released from polymers: diffusion and elimination in brain tissue. *Chem Eng Sci* 46(10):2429–2444.
- Sellke FW, Laham RJ, Edelman ER, Pearlman JD, Simons M. 1998a. Therapeutic angiogenesis with basic fibroblast growth factor: technique and early results. *Ann Thorac Surg* 65(6):1540–1544.
- Sellke FW, Tofukuji M, Laham RJ, Li J, Hariawala MD, Bunting S, Simons M. 1998b. Comparison of VEGF delivery techniques on collateral-dependent microvascular reactivity. *Microvasc Res* 55(2):175–178.
- Sperinde GV, Nugent MA. 2000. Mechanisms of fibroblast growth factor 2 intracellular processing: a kinetic analysis of the role of heparan sulfate proteoglycans. *Biochemistry* 39(13):3788–3796.
- Uchiyama H, Hirano K, Kashiwasake-Jibu M, Taira K. 1996. Detection of undegraded oligonucleotides in vivo by fluorescence resonance energy transfer. Nuclease activities in living sea urchin eggs. *J Biol Chem* 271(1):380–384.
- Vlodavsky I, Bar-Shavit R, Ishai-Michaeli R, Bashkin P, Fuks Z. 1991. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? *Trends Biochem Sci* 16(7):268–271.
- Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsbrun M. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc Natl Acad Sci USA* 84(8):2292–2296.
- Wan WK, Lovich MA, Hwang CW, Edelman ER. 1999. Measurement of drug distribution in vascular tissue using quantitative fluorescence microscopy. *J Pharm Sci* 88(8):822–829.
- Wu P, Brand L. 1994. Resonance energy transfer: method and applications. *Anal Biochem* 218:1–13.
- Yaron A, Carmel A, Katchalski-Katzir E. 1979. Intramolecularly quenched fluorogenic substrates for hydrolytic enzymes. *Anal Biochem* 95:228–235.