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Sequential delivery of angiogenic growth factors improves revascularization and heart function after myocardial infarction

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ABSTRACT

Treatment of ischemia through therapeutic angiogenesis faces significant challenges. Growth factor (GF)-based therapies can be more effective when concerns such as GF spatiotemporal presentation, bioactivity, bioavailability, and localization are addressed. During angiogenesis, vascular endothelial GF (VEGF) is required early to initiate neovessel formation while platelet-derived GF (PDGF-BB) is needed later to stabilize the neovessels. The spatiotemporal delivery of multiple bioactive GFs involved in angiogenesis, in a close mimic to physiological cues, holds great potential to treat ischemic diseases. To achieve sequential release of VEGF and PDGF, we embed VEGF in fibrin gel and PDGF in a heparin-based coacervate that is distributed in the same fibrin gel. In vitro, we show the benefits of this controlled delivery approach on cell proliferation, chemotaxis, and capillary formation. A rat myocardial infarction (MI) model demonstrated the effectiveness of this delivery system in improving cardiac function, ventricular wall thickness, angiogenesis, cardiac muscle survival, and reducing fibrosis and inflammation in the infarct zone compared to saline, empty vehicle, and free GFs. Collectively, our results show that this delivery approach mitigated the injury caused by MI and may serve as a new therapy to treat ischemic hearts pending further examination.

1. Introduction

Ischemic heart disease is a leading cause of morbidity and mortality in the United States. In 2010, the estimated direct and indirect cost of heart disease was approximately $200 billion. In that year, myocardial infarction (MI) was prevalent in 7.6 million Americans. Approximately, 15% of the people who experience a heart attack (MI) in a given year will die of it [1]. During MI, insufficient blood supply to a region of the heart muscle (infarct zone) leads to cell death and pathological remodeling which often progresses to heart failure over time [2]. Therapeutic angiogenesis aims to restore blood flow to the affected ischemic heart muscles by new blood vessel formation from existing vasculature [3–5]. Revascularization by pro-angiogenic therapies has thus far failed to provide satisfactory outcomes in clinical trials [6–8]. Bolus injections of single GFs led to limited efficacy because of loss of bioactivity, missing critical signals in the cascade of events that lead to stable angiogenesis, among others. An effective angiogenesis-based therapy can be developed when a comprehensive understanding of angiogenic mechanisms becomes available [8,9]. Repair and regeneration strategies should focus on utilizing the growth factors (GFs) that play vital roles in the process of angiogenesis, as well as the need to administer them spatiotemporally and in bioactive conformations [6,7,10–12].

Many studies have shown that GFs such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and angiopoietin-2 (Ang-2) are key factors in triggering angiogenesis, but these factors alone may result in leaky and immature blood vessels that are susceptible to early regression [13,14]. Other GFs such as platelet-derived growth factor (PDGF) and angiopoietin-1 (Ang-1) help stabilize neovessels [15,16]. Among potential angiogenic candidates, VEGF and PDGF are promising due to their potency, specificity, and cardioprotective roles [5,6,17,18]. VEGF, an endothelial-specific factor, triggers the process through endothelial cell (EC) sprouting, proliferation, migration, and lumen formation, and is thus primarily needed in the first few days of angiogenesis [17,19,20]. After lumenal formation, mural cells are recruited by PDGF to cover the neovessels and provide stabilization; therefore PDGF is required at a later stage of angiogenesis to prevent vessel regression or the formation of aberrant and leaky vessels [15,17]. It has been shown that early-stage angiogenic factors can have antagonistic effects on late-stage factors and vice versa, when present simultaneously [21–23]. Therefore, it appears imperative to sequentially administer these two GFs to imitate their physiological presence during angiogenesis.

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To control the spatiotemporal cues and protect the bioactivity of VEGF and PDGF, we developed a controlled delivery system composed of fibrin gel and a recently developed biocompatible heparin-based coacervate that we characterized in previous reports [24,25]. Fibrin gel, formed through the polymerization of fibrinogen by thrombin, is commercially available and has been used for protein and cell delivery [26]. Complex coacervates are formed by mixing oppositely charged polyelectrolytes resulting in spherical droplets of organic molecules held together noncovalently and apart from the surrounding liquid and have shown potential in sustained protein delivery [24,25,27–34]. VEGF was embedded into the fibrin gel, while PDGF was loaded into the coacervate then embedded into the gel. The coacervate was used to control the release of PDGF based on its affinity to heparin. This system provided a rapid release of VEGF followed by slow and sustained release of PDGF from a single injection. Here we report the effects of sequentially delivered VEGF and PDGF on revascularization and heart function after MI in rats.

2. Materials and methods

2.1. Release kinetics assay

The release assay (n = 3) was performed using 100 ng of $VEGF_{165}$ and 100 ng of PDGF-BB (PeproTech, Rocky Hill, NJ). PDGF coacervate was made by mixing PDGF with heparin first (Scientific Protein Labs, Waunakee, WI), then with the polycation, poly(ethylene arginyl aspartate diglyceride) (PEAD) [27] at PEAD:heparin:GF mass ratio of 50:10:1. Fibrin gel was made by mixing 90 μl of 20 mg/ml fibrinogen solution (Sigma-Aldrich, St. Louis, MO) containing unbound VEGF and the PDGF coacervate with 5 μl of 1 mg/ml thrombin solution (Sigma-Aldrich, St. Louis, MO) and 5 μl of 1 mg/ml aprotonin solution (Sigma-Aldrich, St. Louis, MO). 100 μl of 0.9% saline was deposited on top of the fibrin gel to be collected at 1 h, 16 h, 1, 4, 7, 14, and 21 days. The samples were incubated at 37 °C. After centrifugation at 12,100 *g* for 10 min, the supernatant was aspirated and stored at −80 °C to detect the amount of released GFs by ELISA kits (PeproTech, Rocky Hill, NJ). The absorbance at 450/540 nm was measured by a SynergyMX plate reader (Biotek, Winooski, VT). Normalizing standards (n = 3) were prepared using the same amounts of free GFs in 100 μl of 0.9% saline.

2.2. Smooth muscle cell chemotaxis assay

Chemotactic media was prepared as 500 μl MCDB-131 + 10% fetal bovine serum (FBS) per well in a 24-well plate with group-specific addition of saline (basal media), empty vehicle, or 100 ng free PDGF or in the coacervate. 8 μm pore size culture inserts (BD Falcon, Franklin Lakes, NJ) were placed in each well and 10^4 baboon smooth muscle cells (SMCs) were pipetted into the insert in 200 μl basal media and plate was incubated at 37 °C. After 12 h, cells remaining inside the insert were removed from the upper surface of the membrane with a cotton swab. Cells that had migrated to the lower surface of the membrane were then fixed in methanol for 15 min. Cells were incubated for 15 min in the dark with PicoGreen fluorescent dye from Quant-iT PicoGreen dsDNA Kit (Molecular Probes, Eugene, OR), diluted 200-fold to working concentration in DPBS. Cells were imaged with a fluorescent microscope (Eclipse Ti; Nikon, Tokyo, Japan) and images were taken in the center of each well in three wells per group and counted manually.

2.3. Endothelial and smooth muscle cell proliferation assays

Human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, VA) or baboon SMCs were seeded at 10^4 cells per well in a 96-well plate and cultured in EGM-2 media (Lonza, Walkersville, MD) and MCDB131 + 0.2% FBS media, respectively. Group-specific additions were made to media with GF concentrations at 20 ng/ml per well for SMCs and 25 ng/ml of each GF per well for HUVEC. The plates were incubated for 48 h at 37 °C. 20 μl of pre-prepared BrdU label was then added for 4 h and the proliferation assays were performed according to the kit's instructions (Millipore, Temecula, CA). The absorbance at 450/540 nm was measured by a SynergyMX plate reader. Absorbance proliferation values were normalized to basal media value.

2.4. Ex vivo rat aortic ring assay

Thoracic rat aortae (n = 3 per group) were dissected according to established protocols [35,36], cleaned from fibro-adipose tissue, and cut into approximately 1.5 mm ring segments. Rings were serum-starved overnight in serum-free endothelial basal medium (EBM). Next day, the rings were embedded in the center of a 3D fibrin matrix that contained different treatment groups (GF dose of 250 ng) with luminal axis perpendicular to the bottom of the well in a 24-well plate. 500 μL of EBM was placed on top of the gel. Rings were incubated at 37 °C for 6 days. Rings were then imaged using brightfield (BF) microscopy and quantified in terms of microvasculature sprouting area in 3 wells per group.

2.5. Rat acute myocardial infarction model

University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) approval was obtained prior to beginning all animal studies. MI and injections were performed as previously described [37]. Briefly, 6–7-week-old male Sprague–Dawley rats (Charles River Labs, Wilmington, MA) were anesthetized with isoflurane (Butler Schein, Dublin, OH), intubated, and connected to a mechanical ventilator. The ventral side was shaved and a small incision was made through the skin. The muscle and ribs above the heart were separated. The heart was exposed and MI was induced by ligation of the left anterior descending (LAD) coronary artery using a 6-0 polypropylene suture (Ethicon, Bridgewater, NJ). Five minutes after the induction of MI, 100 μl of saline, empty vehicle, free VEGF + PDGF (1.5 μg of each GF), or sequentially delivered VEGF + PDGF (using fibrin gel-coacervate system) solutions were injected intramyocardially at 3 equidistant points around the infarct zone using a 31 G needle (BD, Franklin Lakes, NJ). For injections of fibrin gel, thrombin was added to fibrinogen solution and injected shortly before gelation. The chest was closed and the rat was allowed to recover. After 4 weeks, all animals were sacrificed and hearts were harvested for histological and immunohistochemical evaluation.

2.6. Echocardiography

Echocardiography was performed 2 days before surgery (baseline) and at 2, 14, and 28 days post-MI surgery to evaluate cardiac function. Short-axis videos of the left ventricle (LV) by B-mode were obtained using a Vevo 770 high-resolution in vivo micro-Imaging system (Visual Sonics, Ontario, Canada). End-systolic area (ESA) and end-diastolic area (EDA) were measured using NIH ImageJ 1.46r and fractional area change (FAC) was calculated as: [(EDA − ESA) / EDA] * 100%. Percent improvements of one group over another were calculated as the difference between the % drops in FAC values of the first and second groups divided by the higher % drop of the two groups.

2.7. Histological analysis

At 4 weeks post-infarction, rats were sacrificed by injecting 2 ml of deionized (DI) water saturated with potassium chloride (KCl) (Sigma-Aldrich, St. Louis, MO) in the LV to arrest the heart in diastole. Hearts were harvested and frozen in OCT compound. Specimens were sectioned at 6 mm thickness from the apex to the ligation level with 500 μm intervals. Sections were fixed in 2–4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) prior to all staining procedures.