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The effect of a heparin-based coacervate of fibroblast growth factor-2 on scarring in the infarcted myocardium

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ABSTRACT

Effective delivery of exogenous angiogenic growth factors can provide a new therapy for ischemic diseases. However, clinical translation of growth factor therapies faces multiples challenges; the most significant one is the short half-life of the naked protein. We use heparin and a nontoxic polycation to form an injectable coacervate that protects growth factors and preserves their bioactivities. Here we report the effectiveness of fibroblast growth factor-2 (FGF2) coacervate in reducing scar burden in a mouse myocardial infarction model. The coacervate provides spatial and temporal control of the release of heparin-binding proteins. Coacervate treated animals show lower level of inflammation, fibrosis and cardiomyocyte death in the infarcted myocardium. Histological evaluation indicates that FGF2 coacervate significantly increases the number of endothelial and mural cells and results in stable capillaries and arterioles to at least 6 weeks post injection. Echocardiographic assessment shows that FGF2 coacervate promotes cardiac contractibility and inhibits ventricular dilation, suggesting that the improvement at the tissue level leads to better cardiac functions. On the contrary, identical dosage of free FGF2 shows no statistical difference from saline or vehicle control in histological or functional assessment. Overall, injection of FGF2 coacervate ameliorated the ischemic injury caused by myocardial infarction. The promising data in rodent warrant further examination of the potential of clinical translation of this technology.

1. Introduction

Cardiovascular disease (CVD) has high morbidity and mortality and is a significant burden for the patients and the society. According to the latest statistics, CVD accounts for 1 of every 3 deaths in the US with an estimated annual healthcare cost of $300 billion [1]. With an aging population and the prevalence of obesity and diabetes mellitus, the incidence of CVD will likely increase in the near future. On the other hand, current treatments including medication for blood thinning and blood pressure management, and surgical and stenting procedures to restore blood flow, have little effect on the scar tissue that resulted from the ischemic injury. As a consequence, many MI patients undergo a pathological remodeling of the ventricle that eventually leads to congestive heart failure. New therapeutics that can reduce the cardiac scar burden and repair the damaged tissue are urgently needed.

Pathophysiologically, ischemia is a common symptom associated with CVD. Without treatment, long-term ischemia often leads to irreversible tissue injury and organ dysfunction. Therapeutic angiogenesis aims at inducing neovasculature by delivering angiogenic agents and has been pursued as a potential means to rescue ischemia and improve tissue function [2,3]. After decades of investigation, neovasculature can now be achieved by gene, protein or cell delivery [4]. However, efficacies of most clinical trials on therapeutic angiogenesis are inconclusive, consequently, there is no FDA approved angiogenic agent for humans. Specifically to protein therapy, two major factors contribute to the failure of the clinical trials: (i) inadequate knowledge on blood vessel formation and endogenous responses to the ischemic environment and (ii) insufficient bioactivity of the delivered protein [5,6].

FGF2 is frequently applied to induce neovasculature because of its diverse and comprehensive roles in angiogenesis: (i) FGF2 stimulates the migration, proliferation and differentiation of blood

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vessel-associated cells [7]. (ii) FGF2 enhances signaling of other angiogenic factors including vascular endothelial growth factor (VEGF) and platelet derived growth factor [8]. (iii) A recent study finds that sustained FGF stimulation is required for expression of VEGF receptors on endothelium and its response to VEGF [9]. (iv) Furthermore, FGF signaling is essential to vascular integrity as its inhibition causes disintegration of vasculature [10]. In addition to angiogenesis, FGF2 also shows other therapeutic benefits including ability to reduce cardiomyocyte death and promote differentiation of residential cardiac progenitor cells into cardiomyocytes [11,12]. Therefore, we chose to investigate the therapeutic potential of FGF2 coacervate in reducing cardiac scar burden post MI.

Several clinical trials of FGF2 have been conducted in patients with ischemic heart diseases [13]: a phase I trial demonstrated feasibility and safety of FGF2 treatment on patients undergoing coronary bypass surgery [14]. The following phase II trial suggested that coronary artery bypass grafting combined with FGF2 treatment might be beneficial for myocardial revascularization [15]. Another phase I trial showed the safety of FGF2 by intracoronary infusion of FGF2 up to 0.36 μg/kg without adverse effects in patients [16]. However, the subsequent phase II trial indicated that the treatment was only effective at the 90 day time point and showed no difference from the placebo at the 180 day time point [17]. Overall, FGF2 treatment appears to be safe but the efficacy is not yet demonstrated, which is similar to the results of other angiogenic factors [18].

To achieve more effectiveness in growth factor therapies, we developed a coacervate protein delivery platform that consists of a polycation and heparin to deliver heparin-binding proteins [19]. In vitro experiments revealed several advantages of this platform including high affinity to heparin-binding growth factors, ability to control their release in a steady fashion and well-maintained bioactivity of the released factors [20]. Subcutaneous injection of FGF2 coacervate induced substantial and mature vessel formation and exhibited higher bioactivity than naked FGF2 [21]. These laid the groundwork for the current study on the effectiveness of FGF2 coacervate in a mouse model that mimicked human acute myocardial infarction (MI). The hypothesis of this study was that high bioactivity of FGF2 coacervate can effectively reduce the impact of ischemic injury and improve cardiac function post-MI.

2. Materials and methods

2.1. Preparation of [PEAD:heparin] coacervate

Poly(ethylene argininylaspartate diglyceride) (PEAD) was synthesized as previously described [22]. Briefly, ethylene glycol diglycidyl ether and t-BOC protected aspartic acid were polymerized through a polycondensation reaction followed by removal of t-BOC to yield the intermediate, poly(ethylene aspartate glyceride). Conjugation of t-BOC arginine to poly(ethylene aspartate glyceride) was performed by a standard carbodiimide coupling reaction and subsequent removal of t-BOC obtained PEAD. To make the delivery vehicle: [PEAD:heparin] coacervates, PEAD and heparin were dissolved in normal saline and then mixed at room temperature.

2.2. Rhodamine labeling of bovine serum albumin

NHS-rhodamine (Thermo Scientific, Waltham, MA) was utilized to label bovine serum albumin (BSA) (EMD Millipore, Billerica, MA) according to the procedure provided by the vendor. Briefly, NHS-rhodamine was added in BSA solution and reacted at 4 °C for overnight. A dialysis column was used for removal of unreacted agents and purification of the product, rhodamine-BSA.

2.3. Mouse acute myocardial infarction model and intramyocardial injection

Male Balb/cJ mice (Jackson Laboratory, Bar Harbor, ME) with an average age of 10 weeks were used and cared for in compliance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. MI and intramyocardial injections were performed as we previously reported [23]. Briefly, MI was induced by ligation of the left anterior descending coronary artery. 5 min after the induction of MI, 35 μl of saline, delivery vehicle (500 μg of PEAD and 100 μg of heparin) (Scientific Protein Labs LLC, Waunakee, WI), free FGF2 (500 ng of FGF2) (PeproTech, Rocky Hill, NJ) or FGF2 coacervate (500 μg of PEAD, 100 μg of heparin and 500 ng of FGF2) was injected at three sites of the ischemic myocardium (one at the center and two at the border zone of the infarct). For distribution of rhodamine-BSA, free rhodamine-BSA (3 μg of rhodamine-BSA) or rhodamine-BSA coacervate (500 μg of PEAD, 100 μg of heparin and 3 μg of rhodamine-BSA) was injected at the center of the infarct. The surgeon performing the surgical procedures and injections was blinded to the content of the injectant.

2.4. Echocardiography

Echocardiography was performed by a blinded investigator repeatedly before surgery and at 2 and 6 weeks post-infarction to assess cardiac function. Briefly, the heart and respiratory rates were continuously monitored while the body temperature was maintained at 37 °C by a hot pad. Echocardiographic parameters were measured 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 from short-axis images of the LV by B-mode. Fractional area change (FAC) was calculated as: [(EDA−ESA)/EDA] × 100%. Mice died or sacrificed for histological analysis prior to 6 weeks post-injection were not included in the echocardiographic study.

2.5. Histological analysis

At 3 days and 2 and 6 weeks post-infarction, mice were sacrificed and hearts were harvested following the established methods [24]. Harvested hearts were either fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin (H&E) staining or frozen in OCT compound for all other assessments. Specimens were serially sectioned at 8 μm thickness from apex to the ligation level (approximately 0.5 mm in length). For comparison of the ventricular wall thickness in the infarct zone, seven H&E stained slides from each group were randomly selected and the wall thickness of each were measured at two section using NIH ImageJ 1.45s.

For observation of fibrosis, Masson's trichrome kit (IMEB, San Marcos, CA) was used to stain collagen fibers. The area of collagen deposition was measured by ImageJ and the extent of fibrosis was determined by dividing the area of collagen deposition by the entire cardiac tissue area. Twelve randomly selected sections from each group were utilized for quantification.

2.6. Immunofluorescent staining

For evaluation of inflammation, a rat anti-mouse CD68 (Abcam, Cambridge, MA) was used first followed by a Cy3-conjugated anti-rat IgG antibody (Invitrogen, Carlsbad, CA). For detection of endothelial cells, a rat anti-mouse CD31 (BD Biosciences, San Jose, CA) and the Cy3-conjugated anti-rat IgG antibody or a FITC-conjugated anti-VWF antibody (US Biological, Swampscott, MA) was utilized. For α-SMA staining, a FITC-conjugated anti-α-SMA monoclonal antibody (Sigma Aldrich, St. Louis, MO) was utilized. All slides were last counterstained with DAPI (Invitrogen, Carlsbad, CA). For quantification, four to six sections from different hearts were used for each group. For CD68 and CD31 quantification, the number of CD68- and CD31-positive cells was counted and confirmed by the nuclei (DAPI). For VWF and α-SMA quantification, the number of VWF- and α-SMA-positive cells associated with CD31-positive cells was counted and confirmed by nuclei staining (DAPI).

For distribution of rhodamine-BSA, slides were stained with DAPI and observed directly under the microscopy. For quantification, intensity of fluorescence was determined by ImageJ and normalized to the background value.

2.7. Statistical analysis

Results were presented as mean ± standard deviation. One-way ANOVA followed by Tukey's HSD test was used to compare each measurement. P values <0.05 was considered significantly different.

3. Results

3.1. [PEAD:heparin] vehicle offers spatial control of the release of heparin-binding factor

For protein delivery that targets local tissues, it is important to control the spatial distribution of incorporated proteins. To examine the spatial distribution of the injected coacervate, we employed BSA to represent heparin-binding proteins as it was commonly used as a model molecule in heparin-protein binding studies [25,26]. In an aqueous solution, rhodamine-BSA could not be visualized as it dissolved homogenously and the low concentration of rhodamine made it below detection limit under the experimental conditions (Fig. 1b). In contrast the delivery vehicle