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Engineering a growth factor embedded nanofiber matrix niche to promote vascularization for functional cardiac regeneration

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ABSTRACT

The major loss of tissue extracellular matrix (ECM) after myocardial ischemia is a serious burden that gradually leads to heart failure. Due to lack of available treatment methods to restore the cardiac function, various research strategies have come up to treat the ischemic myocardium. However these have met with limited success due to the complexity of the cardiac tissue, which exhibits a nanofibrous collagenous matrix with spatio-temporal localization of a combination of growth factors. To mimic the topographical and chemical cues of the natural cardiac tissue, we have fabricated a growth factor embedded nanofibrous scaffold through electrospinning. In our previous work, we have reported a nanofibrous matrix made of PLCL and PEOz with an average diameter of 500 nm. The scaffold properties were specifically characterized *in vitro* for cardio-compatibility. In the present study, we have loaded dual growth factors VEGF and bFGF in the nanofiber matrix and investigated its suitability for cardiac tissue engineering. The encapsulation and release of dual growth factors from the matrix were studied using XPS and ELISA. Bioactivity of the loaded growth factors towards proliferation and migration of endothelial cells (HUVECs) was evaluated through MTS and Boyden chamber assays respectively. The efficiency of growth factors on the nanofibrous matrix to activate signaling molecules was studied in HUVECs through gene expression analysis. Preclinical evaluation of the growth factor embedded nanofibrous patch in a rabbit acute myocardial infarction (AMI) model was studied and cardiac function assessment was made through ECG and echocardiography. The evidence for angiogenesis in the patch secured regions was analyzed through histopathology and immunohistochemistry. Our results confirm the effectiveness of growth factor embedded nanofiber matrix in restoration of cardiac function after ischemia when compared to conventional patch material thereby exhibiting promise as a valuable therapeutic solution to treat ischemic disorders.

1. Introduction

The ECM of the cardiac tissue is majorly composed of structural and nonstructural proteins [1]. The structural proteins majorly include collagens, fibronectin, elastin and laminins, which are primarily involved in building the framework for the tissue, whereas the non-structural glycoproteins like glycosaminoglycans (GAGs) and proteoglycans help in maintaining the tissue homeostasis through facilitating growth factor signaling. Collagen type I has extensive load bearing capacity by nature, which can support the heavy stress generated in cardiac tissue. In the cardiac tissue, collagen III and V weave around collagen I bundles and cardiomyocytes forming a reticulate network [2]. Fibronectins present in cardiac tissue not only assist in maintaining the structure but also provide binding sites for growth factors (basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), Bone morphogenic protein (BMP1) and their receptors [3]. Elastin in the form of insoluble elastic fibers provides resilience to the cardiac tissue during systole and diastole. The laminins establish firm contact to the cells through the cell surface integrins and protect the heart from anoikis [3]. GAGs confer viscoelastic property to cardiac tissue. Due to this property, the GAGs nullify the pressure exerted during mechanical overload in cardiac tissue. Among the GAGs, heparin sulfate has strong

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affinity to VEGF, bFGF and HGF mediated through α-L-iduronic acid moiety [4]. From the literature it is clear that growth factor embedded nanofibrous networks are the dominant entities in the functional cardiac ECM. Mimicking this complex bioactive topography through the use of synthetic scaffolds and growth factors is a serious challenge faced by the tissue engineering community. Tailormade synthetic biodegradable polymeric scaffolds with defined topography and mechanical stiffness to match ventricular tissue properties have been explored recently [5]. The nanofibrous scaffolds have potential to replace the ischemic cardiac tissue structurally, but in order to reestablish the native cellular contacts the presence of growth factors in the synthetic matrix is indispensable [6]. During myocardial infarction, the degradation of collagen matrix by the inflammatory proteases leads to cellular detachment followed by necrosis [6]. Although GAGs perform a regulatory function during tissue homeostasis, they are prone to lysosomal degradation and induce inflammatory response during ischemia [7]. The inflammatory cytokines secreted by the necrotic cells helps to recruit the macrophages at the site of damaged tissue. The invading macrophages engulf the cellular remnants and tissue debris to form the granulation tissue followed by left ventricular (LV) remodeling [8] [9]. The LV remodeling promotes the maturation of scar tissue and results in gradual deterioration of cardiac function thereby leading to organ failure [10].

Growth factors are proteins, that belong to either chemokine or cytokine family and have been shown to stimulate and amplify the cell signaling process [6]. The commonly used growth factors for angiogenesis are VEGF and bFGF [11]. Among VEGF isoforms (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF 206), VEGF121 and VEGF165 are freely diffusible with high mitogenic activity [12]. Similarly FGF1, FGF2 or bFGF exhibit chemokine and angiogenic properties among the 21 isoforms [13]. The VEGF and FGF function through vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR) present on the cell surface during blood vessel formation [8] [9]. In addition, bFGF stimulates VEGFR2, platelet derived growth factor receptor (PDGFR) and VE-Cadherin expression for neovascularization, blood vessel stabilization and maintenance [14]. Thus FGF stimulates angiogenic signaling in a VEGF-dependent manner.

Growth factor supplementation through polymeric scaffolds has emerged as a novel multifaceted approach in cardiac regenerative medicine [6]. Scaffolds (film) made of biodegradable polycaprolactone (PCL), when crosslinked with natural ECM derivative heparin strengthen the VEGF binding ability [15]. Similarly hydrogel scaffolds of poly (ethylene glycol) (PEG) crosslinked with heparin enhanced the immobilization of VEGF and FGF [16]. Thermoresponsive hydrogel scaffolds of poly(valerolactone) poly(-ethyleneglycol) poly(valerolactone) conjugated with VEGF improved the cardiac functional recovery in rat AMI model [17]. A fusion protein consisting of VEGF and collagen binding domain peptide "TKKTLRT" when loaded in collagen membranes enhanced VEGF binding and improved the vascular density in infarcted myocardium [18]. Synergistic effect due to dual growth factors VEGF and FGF was observed when they were loaded in PEG hydrogel scaffolds as compared to their individual forms [11]. Improved vascularization of hind limb ischemia was seen in fibrin poly (ether) urethane-poly (dimethylsiloxane) scaffolds loaded with VEGF and bFGF [19].

We have previously developed heterogeneous nanofibrous scaffolds made of biodegradable polymers poly (L-lactide-co-caprolactone) (PLCL) and poly (2-ethyl-2-oxazoline) (PEOz). The physiochemical and biological properties of the scaffolds have been investigated for its suitability in cardiac tissue engineering applications [20]. In this manuscript, we have extended the study further to explore the possibility of encapsulating angiogenic growth factors into nanofibrous scaffolds to mimic the native tissue architecture. The functional bioactivity of the growth factors was tested *in vitro* using chemical hypoxia model. *In vivo* evaluation of the bioactive patch, as a therapeutic alternative to PTFE was tested in a rabbit model of acute myocardial infarction. The overall hypothesis of the study is to test synergistic effect of topographical (nanofibers) and chemical (growth factors) signals when supplemented via single scaffold system to improve functional cardiac repair and regeneration.

2. Materials and methods

2.1. Materials

PLCL with molar ratio of 50:50 was purchased from BMG Inc, Japan. The PEOz with a molecular weight of 500,000 Da and the solvents 1,1,1,3,3,3 hexafluoro2propanol (HFIP), dichloromethane (DCM) were procured from Sigma Aldrich, India. Recombinant VEGF, bFGF and their respective ELISA development kits were purchased from Peprotech, USA. Human Umbilical Vein Endothelial Cells (HUVECs) (Passage 4) was used to study the bioactivity of the loaded growth factors. The endothelial basal medium (EGM 2) and growth factor supplementation (EGM bullet kit) was obtained from Lonza, USA. Phosphate buffered saline (PBS), Fetal bovine serum (FBS) were purchased from Gibco, USA.

2.2. Methods

2.2.1. Fabrication of growth factor loaded scaffolds

The growth factors VEGF and bFGF independently as well as dual forms were loaded in fibrous scaffolds of PLCL and B73 (physical blend of PLCL and PEOz in the ratio 7:3) during electrospinning. The concentration of growth factors used for the study was based on published reports [21]. Briefly 1 μg/mL VEGF and bFGF stocks were prepared, from which 80 μL containing 80 ng of VEGF was made up to 250 μL using PBS. Similarly 56 μL containing 56 ng of FGF was made up to 250 μL using PBS. For dual growth factor loading 80 μL of VEGF and 56 μL of bFGF were made up to 250 μL using PBS. The growth factor mixture was dissolved in 5 mL of polymer solution individually as well as dual forms and electrospun at an applied voltage of 15 kV, flow rate of 0.013 mL/min, tip-to-target distance of 10 cm and needle gauge size of 26 G. The fibers were collected on an aluminum foil and used for *in vitro* and *in vivo* studies.

2.2.2. Release profile of growth factors

Initially, the amount of growth factors present in the scaffolds was estimated using ELISA. 30 mg of scaffold loaded with growth factors was dissolved in 1 mL DCM:PBS (50:50) and vortexed for one minute followed by centrifugation at 6000-8000 rpm for 5 min. The supernatant was carefully aliquoted and analyzed using ELISA kit for VEGF and bFGF quantification as described earlier [22]. For determining the release profiles of the growth factors from the scaffolds, 30 mg of scaffolds with and without growth factors in quadruplets ($n = 4$) were placed in 24 well plates. 1 mL of PBS (pH 7.4) was added to the wells and kept in a shaking water bath at 37 °C. The proteins released in the supernatant were collected at regular time intervals (0.25 h, 0.5 h, 0.75 h, 1 h, 1.25 h, 1.5 h, 1.75 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 5 h, 6 h, 7 h, 8 h, 10 h, 12 h, 16 h, 20 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h and 240 h) and replaced with fresh PBS. The collected samples were quantified using ELISA for *in vitro* bioactivity assessment [22].

2.2.3. Growth factors quantification by ELISA

The samples were quantified for the presence of VEGF and FGF using ELISA development kit as per manufacturer's protocol. Briefly