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VEGF-B electrotransfer mediated gene therapy induces cardiomyogenesis in a rat model of cardiac ischemia

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ABSTRACT

Atherosclerosis induced myocardial infarction (MI) continues to be a major public health concern. Regenerative therapies that restore cardiac muscle cells are largely absent. The rate of cardiomyogenesis in adults is insufficient to compensate for MI damage. In this study, we explored the capacity of a gene therapy approach to promote cardiomyogenesis. We hypothesized that VEGF-B, critical during fetal heart development, could promote cardiomyogenesis in adult ischemic hearts. Gene electrotransfer (GET), a physical method of in vivo gene delivery, was adapted to the rat model of MI. Favorable pulsing parameters were then used for delivery of pVEGF-B and compared to a sham control in terms of infarct size, cardiomyocyte proliferation and presence of new cardiomyocytes. Ki67 immunoreactivity was used for proliferation analysis. Newly synthetized DNA was labeled with BrdU to identify new cells post-infarction. Cardiac troponin co-localization indicated proliferating and new cardiomyocytes histologically. Eight weeks post-treatment, GET pVEGF-B treated hearts had significantly smaller infarcts than the sham control group ($p < 0.04$). Proliferating and new cardiomyocytes were only present in the GET of pVEGF-B group, and absent in the controls. In summary, GET pVEGF-B promoted cardiomyogenesis post-MI, demonstrating for the first time direct evidence of myocardial regeneration post-infarction.

1. Introduction

Heart disease continues to be a leading cause of death in the United States accounting for one fourth of all annual deaths [1,2]. Congestive heart failure develops in approximately 22% of men and 46% of women surviving myocardial infarction (MI) within six years post-MI [1–3]. The five year survival rate for patients diagnosed with heart failure is only 50% [1–3]. Current clinical therapies aim primarily at direct revascularization to improve the survival rates for patients post-MI [4]. Revascularization strategies aim to alleviate ischemia caused by extensive atherosclerosis blocking coronary vessels causing downstream ischemia in the ventricles of the heart [5]. However, the myocardium does not regenerate or reorganize to its former electrical, histological, and functional pumping potential [1,6]. Post ischemic injury, inflammation and myocyte cell death is followed by cardiac fibroblast proliferation, infiltration and collagen deposition as scar tissue replacing cardiomyocytes. The ischemia-injured myocardium thus consists of thin, non-contracting patches with poor contractile activity, limiting cardiac output [7]. There is little evidence of return of viable cardiomyoctes in patients surviving MI [6–8].

Cardiomyogenesis is the process by which new cardiomyocytes develop either via mechanisms of differentiation of progenitor cells, or via proliferation of existing cardiomyocytes. Cardiac myocyte renewal in adult hearts does occur at a very slow rate, reportedly starting with 1% annual renewal at the age of 20 and declining to 0.3% annual renewal by 75 years [9]. While there is some debate surrounding the exact annual renewal rate [10], what is clear is that the endogenous rate of cardiomyocyte renewal is clinically insufficient to compensate for damage from an infarction. Reports of regenerative therapies for myocardial infarction often examine indirect measures of infarct size and left ventricular wall motion as evidence of myocyte regeneration [11,12], however little direct evidence of cardiomyogenesis has been reported to date. Many growth factors have been documented to promote angiogenesis, cardiomyocyte differentiation and proliferation. One such factor is vascular endothelial growth factor B (VEGF-B). VEGF-B is the predominant form of VEGF in fetal developing heart, yet is absent in adult hearts. It has also been shown to offer cardiac protection to ischemic injury in a rat overexpression model [13–17]. In our current work, we report an original regenerative VEGF-B gene therapy approach that promotes cardiomyogenesis post-myocardial infarction.

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Gene delivery methods to the myocardium vary from viral based to physical delivery methods. In our previous studies, we reported on plasmid DNA delivery via GET to cardiac muscle in vivo in a porcine model [18,19]. GET of plasmid DNA can be applied safely directly to the ischemic and non-ischemic myocardium and gene expression can be modulated by pulsing conditions [18,19]. We have also determined that there may be a moderate therapeutic benefit to local delivery of pVEGF-A to ischemic myocardium, with improved myocardial perfusion two weeks after treatment [19], reduced infarct size, arteriogenesis and overall reduced progression to pathogenic cardiac remodeling seven weeks after treatment [20].

Our current study is focused on the regenerative capacity of GET delivery of VEGF-B encoding plasmid DNA in a rat model of myocardial infarction. We demonstrate that gene expression is enhanced with electrotransfer for up to a week of transient expression. The biodistribution of VEGF-B expression includes the pericardium, and the full thickness of the myocardium and the treatment site. Eight weeks post-treatment pVEGF-B GET group had significantly smaller infarct size, and new and proliferating cardiomyocytes compared to the sham control group that had larger infarct sizes and no proliferating or new cardiomyocytes. Overall, our results indicate a potential long-term regenerative therapeutic benefit of exogenous VEGF-B gene delivery mediated by electrotransfer to ischemic myocardium, with evidence of cardiomyocyte renewal.

2. Materials & methods

2.1. Animals

Sprague-Dawley male rats were purchased from Charles River (Worcester, MA), at an approximate weight of 500 g. All experimental studies followed an approved Old Dominion University's Institutional Animal Care and Use Committee protocol, in accordance with the *Guide for the Care and Use of Laboratory Animals* at an AAALAC-accredited facility. Animals were quarantined and acclimated for a 7-day period before any procedures were conducted.

2.2. Plasmid

Plasmid DNA encoding luciferase, gWizLuc, was purchased from Aldevron (Fargo, ND). Plasmid DNA encoding human VEGF-B_{186} tagged with a DDK tag (pVEGF-B-DDK), and plasmid DNA encoding human VEGF-B_{186} (pVEGF-B) without a tag were purchased from OriGene (Rockville, MD). Plasmid DNA was suspended in sterile saline at 2 mg/ml by Aldevron. Endotoxin levels were <0.1 EU/μg plasmid, confirmed by Aldevron via a *Limulus* Amebocyte Lysate assay.

2.3. Myocardial infarction

Surgical procedure and induction of acute ischemia were performed as previously described [21]. Briefly, animals were anesthetized with 3–4% isoflurane inhalation and intubated. Respiration was maintained with a volume-controlled mechanical ventilator. A three-lead electrocardiogram (Accusync Medical Research Company, Milford, CT) was used for monitoring cardiac activity throughout the procedure. Sterile technique was employed for all surgical procedures. A left thoracotomy was performed to expose the left ventricle. The pericardium was left intact. The left anterior descending (LAD) coronary artery was permanently ligated with a 5–0 silk suture below the second diagonal branch, inducing downstream ischemia. Observation of blanching downstream of the LAD ligation was used to confirm induction of ischemia, if no blanching was observed the ligation was repeated until successful a occlusion was observed.

2.4. Gene electrotransfer

Initial gene electrotransfer experiments were performed to establish the small animal model for electrotransfer gene delivery to a beating heart. Luciferase encoding plasmid DNA (gWizLuc) was used to establish kinetics of gene expression over time. Animals were randomly assigned to either the GET or DNA injection only (IO) without electrotransfer groups, with $n = 4$ for each group. Human VEGF-B encoding plasmid DNA tagged with a DDK tag was used to determine distribution of gene expression post-delivery with $n = 3$. Once gene delivery, kinetics and distribution were confirmed, plasmid DNA encoding the effector gene (human VEGF-B) was delivered via electrotransfer to ischemic myocardium and compared to a sham control of a saline injection for therapeutic outcomes, with $n = 4$ in GET and saline control groups. GET was performed immediately after the onset of ischemia. A 2 mm, 4-needle penetrating electrode was used for to deliver the pulses. The gap between the electrodes was 5 mm. Plasmid DNA or saline injections of 100 μl were administered to the left ventricular myocardium wall. For GET groups, the needle electrodes were then inserted into the myocardial wall around the injection site. A square wave pulsed electric field of 60 V was then applied to the needles, synchronized with the rise of the R-wave of the ECG. Each pulse was 20 ms long. Four pulses were delivered per site. Injection only or saline control groups received no electric pulses. Pulse parameters were selected based on optimal pulse parameters for GET in swine hearts [19,20].

2.5. Immunofluorescence analysis

Distribution of gene delivery was determined by immunofluorescence staining for the DDK tag protein. Hearts were collected two days post-GET, fixed in 4% paraformaldehyde, imbedded in freezing medium and cryo-sectioned. Tissue sections were then stained for immunoreactivity with DDK-tag protein with a mouse monoclonal anti-DDK antibody (TA50011-1, OriGene, Rockville, MD) and labeled with an AlexaFluor546 conjugated goat anti-mouse IgG secondary antibody (ThermoFisher Scientific, Grand Island NY). Negative control samples were treated with secondary antibody only, without primary antibody. Immunofluorescence (IF) imaging was performed with an upright Olympus fluorescence microscope. Pericardium, epicardium, myocardium, and endocardium were imaged. Representative results are presented in Fig. 1A–D.

Histological assessment of cardiomyocyte proliferation and renewal were performed. Cardiomyocyte proliferation was assessed with co-localization of a nuclear proliferation marker, Ki67, and cytoplasmic cardiac troponin I, which together served to identify proliferating cardiomyocytes. New cardiomyocytes were identified with the bromo deoxyuridine (BrdU) proliferation assay. Animals received a BrdU subcutaneous injection on day 0 post-procedure at 5 μg/g of body weight, and were provided BrdU spiked water (0.8 mg/ml) for 14 days. Hearts were collected at the eight-week time point and fixed in 4% paraformaldehyde. Fixed hearts were paraffin embedded and sectioned by IDEXX Laboratories, Inc. (Westbrook, Maine). Sections were deparaffinized in CitriSolv™, and rehydrated in gradient alcohol. Antigen retrieval was performed in citric acid (pH 6). Sections were then stained for immunoreactivity with an anti-Ki67 antibody (ab15580, Abcam, Cambridge, MA) and an anti-Cardiac Troponin I antibody (ab19615, Abcam, Cambridge, MA); or for immunoreactivity with an anti-BrdU antibody (ab115874, Abcam, Cambridge, MA) and an anti-Cardiac Troponin T antibody (ab45932, Abcam, Cambridge, MA). Ki67 was then labeled with an AlexaFluor546 conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher Scientific, Grand Island NY). Cardiac Troponin I was labeled with an AlexaFluor488 conjugated goat anti-mouse IgG secondary antibody (ThermoFisher Scientific, Grand Island NY). BrdU was then labeled with an AlexaFluor448 conjugated goat anti-mouse IgG secondary antibody (ThermoFisher Scientific, Grand Island NY). Cardiac Troponin T was labeled with an AlexaFluor546