TISSUE ENGINEERING APPROACHES FOR HEART REPAIR— *PRECLINICAL STUDIES IN SMALL AND LARGE ANIMALS*

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ABSTRACT

Angiogenic therapy is a promising approach for tissue repair and regeneration. However, recent clinical trials using protein delivery or gene therapy to promote angiogenesis have failed to provide therapeutic effects. A key factor for achieving effective revascularization is the durability of the microvasculature and the formation of new arterial vessels. Accordingly, we carried out experiments to test if intramyocardial injection of self-assembling peptide nanofibers (NF) combined with vascular endothelial growth factor (VEGF) could create an intramyocardial microenvironment with prolonged VEGF release to improve post-infarct neovascularization in rats. Our data showed that when injected with NF, VEGF delivery was sustained within the myocardium for up to 14 days, and the side effects of systemic edema and proteinuria were significantly reduced to the same level as control. NF/VEGF injection significantly improved angiogenesis, arteriogenesis, and cardiac performance 28 days after myocardial infarction. NF/VEGF injection not only allowed controlled local delivery, but also transformed the injected site to a favorable microenvironment that recruited endogenous myofibroblasts and helped achieve effective revascularization. Strikingly, the engineered vascular niche further attracted a new population of cardiomyocyte-like cells to home to the injected sites, suggesting cardiomyocyte regeneration. Follow-up studies in pigs also revealed healing benefits consistent with observations in rats. In summary, this study demonstrates a new strategy for cardiovascular repair with potential for future clinical translation.

Key words: Myocardial infarction; Cell therapy; Tissue engineering; Vascular endothelial growth factor

INTRODUCTION

Cumulative studies have revealed that it is possible to utilize nanotechnology and biomaterials to recapitulate biomimic milieu and manipulate cell activities *in vitro*. Such innovation has opened a new field in regenerative medicine far beyond the original scope of using biomaterials merely for controlled drug release. Accordingly, how to create an engineered niche *in vivo* that stimulates the tissue regeneration capability is an emerging and imperative question. For example, Martino *et al.* recently demonstrated the creation of an engineered microenvironment for bone healing *in vivo*, which recruits growth factors to the wound and convinces cells to repair the damage. However, engineered microenvironments with curative efficacy are rarely successful from "bench to bedside," especially for cardiac tissue repair, due to the complexity of the native tissue construction and morphogenesis.

Self-assembling peptide nanofibers (NF) are short oligopeptides which self-assemble into nanofibrous gel when mixed with salt solution at physiological pH. These unique properties make NF not only slow degradable and low in immunogenicity, but also therapeutically potent for sustained release of a drug or growth factor via noncovalent coupling or covalent bonding. Specifically, recent studies have demonstrated that NF injections give rise to an intramyocardial microenvironment that promotes vascular cell infiltration and maturation. Accordingly, we hypothesized that the injection of VEGF along with NF creates an intramyocardial microenvironment suitable for vascular cell recruitment, proliferation and maturation and would thereby improve post-infarction neovascularization and cardiac performance in rodents.

MATERIALS AND METHODS

All animal procedures were approved by the National Cheng Kung University Institutional Animal Care and Use Committee.

Rat experimental myocardial infarction model

Male Sprague-Dawley rats (~250 g) were anesthetized, and the chest cavity was opened either without coronary artery ligation (sham) or with permanent ligation of the left anterior descending (LAD) coronary artery. Then, a total of 80 μl of each of the various treatment formulations was given by intramyocardial injection divided among 6 different sites at the border zones and the infarcted area of the heart. Cardiac performance was assessed by echocardiography, animals were then sacrificed and their tissues stained for level of pathological remodeling, angiogenesis, and arteriogenesis (Supplementary Methods).

Preparation of NF/VEGF treatments and the *in vivo* release kinetics of VEGF

Self-assembling peptide nanofibers (peptide sequence AcN-RARADADARARADADA-NH2; SynBioSci) were gel-formatted using sterile phosphate-buffered saline (PBS) as a solvent, as described previously. Recombinant human $VEGF_{165}$ was thoroughly mixed with either the NF or PBS at a volume ratio of 1:9 to obtain the required concentration for each experiment. Rat hearts with various treatment were harvested at various time points, and the border and infarct myocardium were trimmed and dissolved in 400 μl of nonreducing buffer containing 1% Triton X-100, 50 mM Tris (pH 7.4), 300 mM NaCl, 5 mM EDTA, and 0.02% NaN_3, supplemented with a proteinase inhibitor cocktail (Sigma-Aldrich) at a 1:200 dilution for protein extraction. The soluble protein extractions were then subjected to a human $VEGF_{165}$ ELISA assay (eBiosource).

Exogenous bone marrow cell injection study

At 7 days post-treatment, rats were intravenously injected with 10^7 rat BMCs, which had been freshly isolated from the tibias and femurs and labeled with DiI (Invitrogen) for tracing. For β2-integrin blockage, 0.5×10^6 cells were incubated with anti-β2-integrin monoclonal antibody (BD Pharmingen) at a concentration of 20 μg/mL for 30 minutes on ice immediately before injection (*54*). The rats were then sacrificed one day after the BMC injection, and each heart was harvested for tissue processing as described above. The myocardial sections were stained with DAPI, and 4 pictures were taken blindly for each section. DiI signal was detected using a red fluorescence filter set at a constant exposure time. Only cells whose nuclei were surrounded with DiI signal were counted as DiI^+ cells.

Cardiomyocyte-specific fate-mapping study

Double-transgenic MerCreMer-ZEG mice were generated as described previously. Tamoxifen (Sigma) was dissolved in sunflower oil (Sigma) at a concentration of 5 mg/mL and was injected intraperitoneally at a dose of 40 μg/g body weight/day for 14 days. The $GFP^{-}cTnI^{+}$ cells were quantified blindly, and the nuclei of the counted cells were surrounded with cTnI but not GFP signal.

Pig model of experimental myocardial infarction

Sexually mature Lanyu minipigs (~5 months old) were induced with MI by a permanent occlusion of mid-LAD coronary artery, immediately followed by injection of 2 ml PBS or 1 % NF with or without 100 ng/ml VEGF into the peri-infarct and infarct area. Cardiac functions were assessed by echocardiography before and immediately after MI and together with hemodynamic measurements through catheterization 4 weeks later.

Pigs were then sacrificed, and hearts were harvested as previously described. In brief, the atria and the right ventricle were removed, and then the LV was cut into two parts at the papillary muscle insertion site and placed upright. Necrotic tissue (pale) was quantified by manual tracing and software calculation (Image J).

Statistical analysis

Differences were determined by two-tailed unpaired *t* test, or by one-way repeated-measures analysis of variance (ANOVA) with Newman-Keuls post-hoc test to compare means between multiple groups, or by two-way ANOVA with subsequent Bonferroni's post-hoc test to compare means between multiple groups on multiple time points. A value of $P < 0.05$ is considered statistically significant.

RESULTS

Intramyocardial NF/VEGF improves cardiac performance in rats post-MI

We used a rat experimental MI model to examine the therapeutic effects of intramyocardial NF/VEGF injection. At 28 days post-MI, the intramyocardial NF/VEGF injection significantly improved cardiac systolic function compared to NF or VEGF alone, as indexed by the left ventricular fraction shortening (LVFS). Correspondingly, the NF/VEGF injection also effectively prevented pathological remodeling and ventricular dilation as evidenced by restrained infarct size and reduced collagen deposition in the non-infarct region. NF/VEGF treatments also decreased end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively) in the left ventricles. In contrast, although there was a marginal dose-dependent amelioration of the LVFS, infarct size, collagen deposition of non-infarct region, LVESD, and LVEDD for the VEGF-treated groups at 28 days post-MI, there was no significant difference between the PBS and VEGF only treatment groups, which indicates that the delivery of VEGF alone was not sufficient to provide cardiac benefits post-MI.

Intramyocardial NF/VEGF injection increases cardiac performance and arteriogenesis post-infarction in pigs

We then performed experimental infarction in an established pig model to test the therapeutic efficacy and the clinical translational potential. PBS, NF only, VEGF only, or 100 ng/ml VEGF was injected immediately after MI. Consistent with the results in rats, VEGF injection only slightly improved cardiac function with no significant difference compared to

control; whereas NF/VEGF injections significantly improved cardiac performance at 28 days post-MI. The reduced infarct size, and hemodynamic parameters such as + dP/dt, – dP/dt, time constant of LV pressure decay and maximum chamber elasticity also showed consistency. Importantly, similar to the outcome in rats, delivery of VEGF with or without NF both improved post-MI angiogenesis, whereas only NF/VEGF injection significantly improved arteriogenesis at 28 days post-MI. The result thus indicates the cardiac benefit relies on post-MI arteriogenesis rather than angiogenesis.

DISCUSSION

Rising reports have revealed the advantage and importance of biomaterials in cardiac tissue engineering. Despite the enthusiasm, to our knowledge, there is only one ongoing clinical trial using material injection for cardiac repair (clinicaltrials.gov identifier: NCT00557531), which may be owing to the relatively lacking evidence in large animal studies serving as "pre-clinical trials". Importantly, we performed large animal study using pig model to demonstrate the translational potential. However, since the immediate post-MI treatment may not be relevant to truly clinical situation, whether this approach also works in the chronic case and whether there exists an optimal therapeutic time window require further examination. The optimization of NF/VEGF dosage and long-term studies are also needed for clinical translation. In addition, although we showed the major merit of NF to build beneficial microenvironments, the exact pivotal cues may be attributed to the biometric fiber diameter, tendency to induce cell adhesion, slow degradability, or a combination of all of them. Therefore, the underlying mechanism as well as the criteria for biomaterial design still requires further investigation. In conclusion, here we report that nanofibers are able to create an *in vivo* microenvironment for cardiovascular regeneration and also provide positive therapeutic effects post-MI in both small and large animals. This strategy not only holds promise for future basic tissue engineering research, but also for translational medicine.

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