Enhanced neovascularure formation in ischemic myocardium following delivery of pleiotrophin plasmid in a biopolymer

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Abstract

Coronary heart disease is currently the leading killer in the western world. Therapeutic angiogenic agents are currently being examined for treatment of this disease. We have recently demonstrated the effective use of Pleiotrophin (PTN) as a therapeutic agent for treatment of ischemic myocardium. We have also shown that injection of the biopolymer fibrin glue preserves left ventricular geometry and prevents a deterioration of cardiac function following myocardial infarction. Due to the low transfection efficiency of naked plasmid injections, we examined the use of PTN plasmid and the biopolymer as a gene-activated matrix in the myocardium. In this study, we demonstrate that delivery of PTN plasmid in fibrin glue increases neovascularure formation compared to injection of the naked plasmid in saline.

Keywords: Angiogenesis; Fibrin; Gene therapy; Cardiac tissue engineering

1. Introduction

Coronary heart disease remains the leading killer of both men and women in the western world [1]. Several pre-clinical and clinical studies have examined the use of angiogenic factors for therapeutic angiogenesis in ischemic myocardium. It is hypothesized that increased blood supply through the delivery of angiogenic growth factors will improve cardiac function following a myocardial infarction. In the initial stage of an infarct, the center portion consists of necrotic tissue while the edges, or borderzone, contain viable cardiomyocytes that are at risk. These at-risk myocytes will eventually become necrotic and produce a larger infarct if a blood supply is not restored to them. Restoring blood supply to the infarct regions through delivery of angiogenic growth factors, a concept known as therapeutic angiogenesis, will salvage the at-risk cardiomyocytes and reduce infarct expansion with the ultimate goal of improving cardiac function and decreasing the morbidity and mortality associated coronary heart disease. Moreover, increasing neovascularure will aid in tissue remodeling and growth, which is limited in ischemic tissue.

Delivery of the most studied angiogenic growth factor, vascular endothelial growth factor (VEGF), has resulted in deleterious effects in the myocardium, such as vascular tumor (angioma) formation [2–4]. Due to these adverse effects and mixed results in clinical trials [5–12], we examined the use of another angiogenic agent, Pleiotrophin (PTN) for its ability to form functional neovasculature in the myocardium. We recently demonstrated that injection of a plasmid encoding the PTN gene-induced neovascularure formation in ischemic rat myocardium without any evidence of angioma formation [13].
Typically, naked plasmid injections result in very low transfection efficiency, possibly due to limited exposure of cells to the plasmid [14]. In order to alleviate this problem, researchers have examined delivering plasmid in a polymer matrix. By encapsulating the plasmid in a polymer, the DNA will be released more slowly as the polymer degrades, thus increasing chances of plasmid–cell interaction. This combination of plasmid DNA and a biodegradable structural matrix carrier has been termed a gene-activated matrix [15]. This technology takes advantage of the normal processes of wound healing. The gene-activated matrix immobilizes the plasmid until endogenous wound healing fibroblasts migrate through the matrix, become transfected, and secrete the therapeutic protein encoded by the plasmid. Unlike with the traditional drug delivery paradigm, in this approach, the target cells find the plasmid (or drug). Several studies have demonstrated the effectiveness of this approach [15–18].

Additional studies in our lab demonstrated that injection of the biopolymer fibrin glue preserves left ventricular geometry and cardiac function following myocardial function [19]. Furthermore, we demonstrated that fibrin glue induces neovascularization formation in ischemic myocardium. Due to our positive results with both PTN plasmid and fibrin glue, we now examine whether the combination of these components will further increase neovascularization formation in ischemic myocardium. Previous studies have demonstrated that use of fibrin glue as a gene-activated matrix improves transfection efficiency in keratinocytes up to 100-fold in vitro [20]. We hypothesized that delivery of PTN plasmid in fibrin glue would increase neovascularization formation in ischemic myocardium compared to injection of PTN plasmid alone by acting as a gene-activated matrix.

2. Materials and methods

The study protocol was approved by the Committee for Animal Research of the University of California San Francisco and was performed in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care.

2.1. Rat myocardial infarction model

A previously described ischemia reperfusion model was used in this study [19,21]. Briefly, Female Sprague-Dawley Rats (225–250 g) were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Under sterile technique, the chest was opened by performing a median sternotomy. A single stitch of 7–0 Ticron suture was tightened to occlude the LAD for 17 min and then removed to allow for reperfusion. The chest was then closed and the animal was allowed to recover for 1 week. This lab has extensive experience with this model and has previously demonstrated that this technique results in an acute infarct size of approximately 30% of the LV with reperfusion [22–24].

2.2. Fibrin glue

Commercially available Tisseel VH fibrin sealant (Baxter) was used in this study. Fibrin glue is a biopolymer formed by enzymatic polymerization of fibrinogen monomers. The two components of the gel (fibrinogen and thrombin) remain liquid prior to combination. The fibrinogen component also contains aprotinin, a fibrinolysis inhibitor, to prevent premature degradation of the matrix. The ratio of fibrinogen to thrombin components was 1:1.

2.3. Plasmids

The 580 base pair human PTN open reading frame (ORF) was isolated by RT-PCR from a human adenocarcinoma cell line (SW13), and was subcloned into the HindIII and XbaI sites of pRC/CMV2 (Invitrogen, Carlsbad, California) to generate pRC/CMV2-PTN. The CMV promoter/enhancer and PTN open readingframe were shuttled from the pRC/CMV2-PTN into pIRES (BD Biosciences Clontech, Palo Alto, California) to generate pCMV-PTN-IRES. The beta-galactosidase (beta-gal) ORF from CMVbeta (BD Biosciences Clontech) was shuttled into pCMV-PTN-IRES to generate pCMV-PTN-IRES-beta-gal-neo. The control beta-gal plasmid was a pCMV-beta-gal plasmid (Invitrogen) which is a similar construct without the PTN gene.

2.4. Injections

One week after MI, either 250 μg PTN plasmid in 50 μl saline (n = 6), 250 μg PTN plasmid in 50 μl fibrin glue (n = 7), 250 μg beta-galplasmid in saline (n = 6), or 250 μg beta-gal plasmid in 50 μl fibrin glue (n = 5), was injected into the ischemic LV according to a previously described procedure [19]. Briefly, the rats were anesthetized and the abdomen was opened from the xiphoid process to a left subaxillar level along the lower rib under sterile technique. The LV apex was exposed via a subdiaphragnostic incision, leaving the chest wall and sternum intact. Rats were randomized to one group and injections were made through a 30-gauge needle into the infarcted area of the LV. In plasmid and fibrin glue injections, 250 μg of plasmid was suspended in 25 μl of the thrombin component of the fibrin glue. The thrombin–plasmid mixture was then simultaneously injected into the myocardium with 25 μl of the fibrinogen component using the supplied Duploject applicator (Baxter), which holds the two components in separate
syringes and provides simultaneous mixing and delivery. The diaphragm was sutured closed after suction of the chest cavity and the abdomen was subsequently closed.

2.5. Microbead perfusion

The rats were first anesthetized with an intraperitoneal injection of 200 μl of 50 mg/ml sodium pentobarbital and perfused with fluorescent microbeads according to a previously described procedure [25]. Briefly, the rats were then injected with 700 μl of 50 μg/ml nitroglycerin in order to ensure vasodilation. After 10 min, the chest was opened and the abdominal aorta was cannulated with P-50 tubing. The left atrial appendage was cut to allow for drainage. 9 ml of saline was then perfused retrograde through the heart for approximately 1 min. A 6 ml suspension of 0.2 μm fluorescent carboxylate-modified polystyrene beads (FluoSpheres, Molecular Probes) diluted 1:6 with PBS was then perfused through the heart. The hearts were immediately harvested, rinsed with PBS, and fresh frozen in O.C.T. freezing medium. They were then sectioned into 10 μm slices and examined under a Nikon TE 300 fluorescent microscope. Infarct areas were visualized by noting the paucity of microbeads in the area.

2.6. Histology and immunohistochemistry

Sections were stained with hematoxylin and eosin (H&E) to determine the location of the infarct. Five slides equally distributed throughout the infarct were stained for arterioles and another five slides were stained for capillaries according to a previously described procedures [26]. Briefly, the slides were stained with an anti-smooth muscle actin antibody (Dako; 1:75 dilution) to label arterioles [27]. In order to visualize labeled arterioles and skeletal myoblasts, sections were incubated with a Cy-3 conjugated anti-mouse secondary antibody (Sigma; 1:100 dilution). Arterioles in each section were quantified using the following criteria: (1) positive for smooth muscle labeling, (2) within or bordering the infarct scar, (3) having a visible lumen and (4) a diameter between 10 and 100 μm. The scar area was measured using SPOT 3.5.1 software and arteriole densities were calculated. To stain for capillaries, sections were incubated with biotinylated Griffonia simplicifolia lectin (GSL-1; Vector Labs). Sections were then incubated with peroxidase conjugated streptavidin (LSAB2 System, HRP, Dako) and capillaries were visualized using 3,3’-diaminobenzidine chromagen solution (LSAB2 System). Five high magnification fields within the infarct of each section were chosen at random, capillaries were counted, and vessel density was calculated.

2.7. Statistical analysis

Data is presented as mean ± standard deviation. Differences in vessel densities were compared using a one-way ANOVA with Holm’s adjustment. Significance was accepted at P < 0.05.

3. Results

3.1. Neovasculature formation

To determine if delivery of PTN plasmid in fibrin glue enhances neovasculature formation compared to delivery of the naked plasmid, contiguous sections of ischemic myocardium injected with either PTN plasmid in saline or PTN plasmid in fibrin glue were analyzed and the density of capillaries and arterioles was determined 5 weeks following injection. To ensure that an increase in vessels was due to the activity of PTN plasmid and not the dilution of fibrin glue caused by addition of plasmid to the mixture, these groups were also compared to animals which were injected with β-gal plasmid in fibrin glue. Animals were also injected with β-gal plasmid in saline to serve as a baseline control. Five weeks following injection, the arteriole density within the infarct scar of those animals injected with PTN plasmid was significantly greater than those animals who were injected with PTN plasmid in saline (P = 0.003) (Fig. 1). Arteriole density increased to 18 ± 4 arterioles mm⁻² when PTN plasmid was delivered in fibrin glue compared to 10 ± 2 arterioles mm⁻² when

![Fig. 1. Increased neovasculature formation. Anti-smooth muscle actin labeled arterioles. Note the increased arteriole density in the section from the animal injected with PTN plasmid in fibrin glue (A) compared to those animals injected with PTN plasmid in saline (B), β-gal plasmid in fibrin glue (C), and β-gal plasmid in saline (D).](image-url)
the same plasmid was delivered in saline. Delivery of PTN plasmid in fibrin glue results in approximately an 80% increase in arterioles. The arteriole density in the PTN plasmid in fibrin glue group was also significantly higher than that of the β-gal plasmid in fibrin glue (P = 0.02) and saline groups (P = 0.00009). The arteriole density of those animals injected with β-gal plasmid in fibrin glue was 11 ± 3 arterioles mm⁻² while those animals injected with β-gal plasmid in saline had an arteriole density of 5 ± 1 arterioles mm⁻². In this case, there was approximately a 60% and 70% increase, respectively, in arterioles. There was no significant difference between the animals injected with PTN plasmid in saline compared to those injected with β-gal plasmid in fibrin glue (P = 0.46) (Fig. 2); however, the arteriole density in both groups was significantly greater than those animals injected with β-gal plasmid in saline (P = 0.001 and 0.003, respectively). There were also no significant differences in capillary density within the infarct among the three groups (P = 0.61).

3.2. Perfusion

To determine if vessels induced by treatment injections are functionally connected with pre-existent vessels, fluorescent microbeads were injected into the coronary vasculature via retrograde infusion through the abdominal aorta. Microbeads were visualized in vessels within the infarct area of the hearts injected with PTN plasmid in saline, β-gal plasmid in fibrin glue, PTN plasmid in fibrin glue, and to a lesser extend with β-gal plasmid in saline, thus indicating all treatments produce functional vessels. This also indicates that the increased vessels due to injection of PTN in fibrin glue are connected to the existing coronary vasculature (Fig. 3).

4. Discussion

As anticipated, the results of this study indicate that delivering PTN plasmid in fibrin glue increases neovascularure formation compared to injection of either PTN plasmid or fibrin glue alone. Fibrin glue alone is also capable of inducing a similar response as PTN plasmid alone. Moreover, these newly formed vessels are functionally connected with the existent coronary vasculature. This data further indicates that fibrin glue can be used as an effective gene-activated matrix in the heart. Previous work demonstrated that fibrin glue is an effective matrix for increasing transfection efficiency of keratinocytes in vitro and in rat skin in vivo [20]. Andree and colleagues demonstrated up to a hundred-fold increase in transfection in vitro using human endothelial growth factor (hEGF) plasmid and fibrin glue. When this gene-activated matrix was implanted in vivo, they witnessed a 180 fold increase in EGF expression [20]. Gene-activated matrices are also known to be effective in plasmid gene transfer in other tissues including bone [15,16], skin [17], tendon and ligament [14], and skeletal muscle [18]. This technology allows for the passive

Fig. 2. Arteriole density. Arteriole density in the infarct following injection of PTN plasmid in fibrin glue (FG) was significantly increased compared to injection of PTN plasmid in saline, β-gal plasmid in saline and β-gal plasmid in fibrin glue. Note that naked PTN plasmid did not produce significantly more arterioles than injection of β-gal in fibrin glue. *p<0.05.

Fig. 3. Microbead perfusion. (A) Anti-smooth muscle actin stained arterioles in infarct area after injection of PTN plasmid in fibrin glue. (B) Microbead perfusion through the same section. High density of microbead perfused capillaries in normal myocardium can be observed at the far left of the section. Other perfused vessels are seen in the infarct area. (C) Overlay of A and C demonstrates that vessels formed as a result of injection of PTN plasmid in fibrin glue are functionally connected to existent coronary vasculature.
targeting of endogenous repair cells, primarily fibroblasts. Bonadio and colleagues have reported that 30–50% of available wound cells are transfected within three weeks after gene-activated matrix implantation [14]. Transfection rates following injection of naked plasmid typically peak at 1 week following delivery. Delivery of PTN plasmid in the fibrin glue gene-activated matrix may therefore shift the transfection rate profile, resulting in increased vessel formation.

There also exists the possibility that there is a synergistic effect with fibrin glue and the exogenous PTN protein produced from transfection cells. Fibrin glue is known to act as a sustained release reservoir for PTN protein produced from transfected cells. Fibrin synergistic effect with fibrin glue and the exogenous rate profile, resulting in increased vessel formation.

5. Conclusions

Delivery of PTN plasmid in fibrin glue results in an increase of neovasculature in ischemic myocardium compared to delivery of either component alone. This combination treatment may therefore be a potential treatment for ischemic heart disease in humans.

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References


