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Human Erythropoietin Gene Delivery for Cardiac Remodeling of Myocardial Infarction in Rats

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Abstract

Considerable efforts have been made to exploit cardioprotective drugs and gene delivery systems for myocardial infarction (MI). The promising cardioprotective effects of recombinant human erythropoietin (rHuEPO) protein in animal experiments have not been consistently reproduced in clinical human trials of acute MI; however, the mechanisms underlying the inconsistent discrepancies are not yet fully understood. We hypothesized that the plasmid human erythropoietin gene (phEPO) delivered by our bioreducible polymer might produce cardioprotective effects on post-infarct cardiac remodeling. We demonstrated that intramyocardial delivery of phEPO by an arginine-grafted poly(disulfide amine) (ABP) polymer in infarcted rats preserves cardiac geometry and systolic function. The reduced infarct size of phEPO/ABP delivery was followed by decrease in fibrosis, protection from cardiomyocyte loss, and down-regulation of apoptotic activity. In addition, the increased angiogenesis and decreased myofibroblast density in the border zone of the infarct support the beneficial effects of phEPO/ABP administration. Furthermore, phEPO/ABP delivery induced prominent suppression on Ang II and TGF- β activity in all subdivisions of cardiac tissues except for the central zone of infarct. These results of phEPO gene therapy delivered by a bioreducible ABP polymer provide insight into the lack of phEPO gene therapy translation in the treatment of acute MI to human trials.

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1. Introduction

Despite remarkable advances in guideline-based pharmacologic and interventional treatment over the last two decades, MI is the leading cause of morbidity and mortality worldwide [1, 2]. The post-infarcted heart undergoes a series of structural changes, termed left ventricular (LV) remodeling, at the organ, cellular, and molecular levels, with three overlapping phases: the inflammatory phase, the proliferative phase, and the healing phase [3–5]. Although cardiac remodeling is initially an adaptive response to maintain normal cardiac function, it gradually becomes maladaptive and can lead to adverse clinical outcomes, including heart failure (HF), arrhythmia, and mortality [3–6].

Diverse efforts in experimental and clinical trials have been made to investigate cardioprotective strategies aimed at attenuating reperfusion injury, reversing adverse myocardial remodeling, and ultimately improving cardiac systolic function and clinical outcomes [7–9]. During the last two decades, the clinical indications of rHuEPO have been expanded to anemia in diverse clinical categories, including anemic patients with chronic kidney disease [10]. Beyond the conventional effect of secreted erythropoietin from the kidney in response to hypoxic stimuli, EPO was recently identified as a pleiotropic and organ-protective cytokine, mediating repair and regeneration via anti-apoptosis, anti-inflammation, anti-oxidation, pro-angiogenesis and re-endothelialization, vascular-protectant, mobilization of endothelial progenitor cells, and recruitment of stem cells into the zone of damage [10–13]. Apart from traditional erythropoietic effects, the pleiotropic organ-protective effects of erythropoietin (EPO) make it a frontline cardioprotective candidate [11]. Higher levels of endogenous EPO have been shown to have protective effects against ischemia-reperfusion (I/R) injury in acute MI in humans [14]. Along with numerous *ex vivo* and *in vivo* studies, some clinical studies with a single rHuEPO administration after the percutaneous coronary intervention showed favorable effects on infarct size, cardiac function, and patient prognosis [11, 15]. However, even though the *in vitro* and *in vivo* data supporting a rHuEPO cardioprotective approach are numerous, recent randomized clinical trials in acute MI patients have reported conflicting data [13, 15–17].

The development of drug delivery systems (DDS) has provided new perspectives for the modification of pharmacokinetics and biodistribution of associated genes and proteins by controlling the release rates of therapeutics [18–20]. Recently, we developed a bio-reducible ABP polymer, retaining the unique properties of reductive disulfide linkers coupled with the advantage of arginine residues to enhance cell penetration [21]. In addition, we reported greatly enhanced *in vitro* transfection efficiency and very low cytotoxicity, as well as increased *in vivo* erythropoietic effects over a 60-day period after a single systemic injection of phEPO/ABP polyplexes [22, 23].

To date, little is known about how polymer-mediated phEPO therapy, when compared with naked phEPO gene or rHuEPO protein-alone, distinctly alters cardiac remodeling in the rat MI model. Here, we hypothesized that the sustained release of intramyocardial phEPO gene therapy delivered by ABP polymer might restore heart function and limit pathological cardiac remodeling after MI. Additionally, the present study assessed the effect of phEPO/ABP gene therapy on cardiac remodeling by evaluating the pro-fibrotic angiotensin II (Ang II) and TGF- β expression in rat hearts.

2. Materials and methods

Detailed protocols are provided in the Supplementary material online.

2.1. Preparation of phEPO/polymer polyplexes

The pCMV-hEPO DNA (phEPO) (4,578 bp) was constructed and purified as previously described [22, 23]. Briefly, phEPO and GFP pDNA (gWiz-GFP, Aldevron) were purified with an endotoxin-free plasmid DNA purification NucleoBond® Xtra Maxi plus EF kit (Macherey-Nagel Inc.). The arginine-grafted bioreducible poly(cystaminebisacrylamide-diaminohexane) ABP polymer was synthesized as previously described [21]. Branched poly(ethyleneimine) (bPEI, 25 kDa, Sigma-Aldrich) and rHuEPO protein (Aropotin®, TS Corporation) were used as positive controls. The 50 µg phEPO/ABP polyplexes at a weight ratio of 1:5 were prepared in a 20 mM HEPES/5% glucose buffer with a final volume of 100 µl.

2.2. Myocardial infarction model

MI was induced in 7–8-week-old male Sprague-Dawley (SD) rats (220–250 g) by surgical occlusion of the left anterior descending (LAD) coronary artery as previously described [9]. Briefly, under the mechanical ventilation, the LAD coronary artery was ligated for 60-min occlusion. Successful ischemia was verified by the blanching of the myocardium and dyskinesia of the ischemic zone, indicating interruption in coronary flow. After 60 min of occlusion, the hemostat was removed, and the temporary suture snare was released for reperfusion. Restoration of normal rubor indicated successful reperfusion of myocardium. Following successful ischemia-reperfusion (I/R), the animals were assigned to one of seven groups: sham thoracotomy, I/R only, injection of rHuEPO, injection of phEPO-alone, injection of phEPO/ABP polyplex, injection of phEPO/PEI polyplex, and injection of GFP plasmid/ABP polyplex. Right after reperfusion, the rats received a total injection volume of 100 µl delivered to four separate intramyocardial sites with three injections to the border zone of the infarct in left ventricle (LVfb) and one injection to the fibrotic central zone in left ventricle (LVf) (Fig. 1C).

2.3. Echocardiography

On post-infarct day 5 and 10 after the intramyocardial injection, echocardiography was performed using a 13 MHz linear probe (GE Vivid 7 pro, GE Medical Systems) in rats lightly anesthetized with isoflurane at 1–2 L/minute and spontaneous respiration.

2.4. Pathological analysis

Serial 4 µm thick sections of rat myocardium were fixed, embedded, and stained with H&E stain. Fibrosis, determined by collagen contents and infarct size were evaluated by Masson's trichrome stain. Next, to evaluate the arteriolar density and the loss of cardiomyocytes after MI, heart sections were immunohistochemically (IHC) stained using α -smooth muscle actin (α -SMA) and cardiomyocyte-specific cardiac troponin T (cTnT) antibody. Finally, apoptosis in the LVfb was expressed as the number of TUNEL-positive nuclei per unit area. Analysis of all images was carried out with NIH ImageJ software (NIH) and Aperio ImageScope (Vista) and randomly chosen within the LVfb by an investigator blinded to the treatment groups.

2.5. Western blotting

Protein extracts and immunoblot analyses were performed as described. Briefly, on postinfarct day 10, the rat hearts were harvested and separated into the LVfb and LVf of the LV wall, interventricular septum (IVS), right ventricle (RV), and atria. The protein extracts from rat heart tissues were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and processed for Western blotting with antibodies against Ang II (AbBiotech), TGF- β (Cell Signaling Technologies), and horseradish peroxidase-conjugated

anti-rabbit secondary antibody. α -Actin (Sigma-Aldrich) was used as a housekeeping protein.

2.6 Statistical analysis

We expressed data as the mean $\pm$ SD or mean $\pm$ SEM where indicated. Comparisons between multiple groups were performed by analysis of variance (ANOVA) followed by Tukey *post hoc* testing. Groups with *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Characterization of phEPO/ABP

Nonviral gene therapy, especially using cationic polymers, provides great potential for human gene therapy due to capacity to carry large nucleic acid loads, biosafety with low immunogenicity, and easy modification [20, 24]. Previously, we evaluated the stability and potency of pDNA/ABP polyplexes in serum and several buffer systems [21, 22]. EPO gene therapy delivered by ABP polymer augments the action of EPO compared to rHuEPO and phEPO-alone by its prolonged stability in serum. This long-term expression is beneficial for treatment of both acute and long-term progressive conditions, such as post-infarct cardiac remodeling. The 50 μ g phEPO/ABP polyplexes at a weight ratio of 1:5 showed an average particle size of 214.6 ± 3.7 nm and a zeta potential of 28.3 ± 0.2 mV (Fig. 1A). The polydispersity index (PDI) and size distribution pattern of phEPO/ABP polyplexes are homogeneously condensed (PDI=0.093) than 50 μ g phEPO/PEI polyplex (w/w=1:1) (PDI=0.162) (Fig. 1A and B).

3.2. phEPO/ABP improves cardiac geometry and LV systolic function

Beyond the conventional erythropoietic activity, EPO was recently identified as a pleiotropic organ-protective cytokine [10–13]. Because of the resolution of myocardial stunning and reperfusion injury, the LV ejection fraction (LVEF), a powerful prognostic parameter improves steeply during the first week after MI [25, 26]. We initially evaluated the effect of intramyocardial phEPO/ABP polyplex injections on the time-dependent LV remodeling of cardiac geometry and function using echocardiography examination, compared with other treatment groups in post-infarcted hearts (Fig. 1C, Fig. 2A and B). On both post-infarct days 5 and 10, the administration of phEPO/ABP polyplexes showed an improved LVEF comparable up to the level of sham thoracotomy group and a significantly preserved LVEF when compared with other treatment groups (Fig. 2A and B). An LVEF increase of $>3\%$ with the administration of β -blockers and ACE-inhibitors has shown reduced morbidity and mortality in patients with acute MI.[27] The observed $\sim 15\%$ improvement of the ejection fraction in the phEPO/ABP group, reaching the reference level of the thoracotomy group, suggests promising potential for early phase clinical trials.

Interestingly, this finding was followed by a conserved IVS thickness during the systolic (IVSs) and diastolic phase (IVSd), nearly up to the level of the thoracotomy group in the phEPO/ABP polyplex injection group on both post-infarct days 5 and 10 than other treatment groups (Fig. 2A and B). In addition, the LV diameters during the systolic (LVDs) and diastolic phase (LVDd) for the phEPO/ABP injection group were remarkably reserved to the level of the thoracotomy group on post-infarct day 10 (Fig. 2B). The post wall thickness of the LV during the systolic and diastolic phase did not reveal any differences between the groups. All of the echocardiographic parameters of the GFP DNA/ABP polyplex injection group were comparable to the I/R-only group, excluding the impact of the ABP polymer itself (data not shown). Collectively, these results imply that phEPO gene therapy delivered by the ABP polymer improves the cardiac geometry and LV systolic

function during post-infarct cardiac remodeling, especially acting on the IVS and preventing LV dilation. In the phEPO/ABP group, the conserved hemodynamic alterations and LV dimension may result in a more favorable prognosis after infarct, preventing post-infarct HF.

3.3. phEPO/ABP ameliorates cardiac fibrosis with a reduced infarct size

In the heart, myocardial fibrosis following the loss of myocardial muscle mass is a common pathological end point including MI [28–30]. Initially, fibrosis through increased interstitial collagens is beneficial to the heart by preventing ventricular dilation; however, the cumulative deposition of collagen results in reduced cardiac function with increased stiffness, and post-infarct morbidity, such as HF [29, 30]. We assessed whether the administration of phEPO/ABP polyplexes during I/R injury had an effect in the suppression of cardiac fibrosis on post-infarct cardiac remodeling by the decrease in collagen contents (Fig. 3A and B). Upon Masson's trichrome staining, the post-infarct fibrotic scar areas with bluish-stained high-collagen contents in the LV were decreased in the phEPO/ABP polyplex injection group compared to the I/R group ($15.6 \pm 6.2\%$ vs $38.0 \pm 9.4\%$; $P < 0.01$; Fig. 3A and B). Quantitative analysis revealed that % fibrosis of phEPO/ABP group is significantly decreased compared with other groups (Fig. 3A and B). This decreased fibrosis accounts for at least a portion of the preserved functional effects of the phEPO/ABP polyplex injection in the postinfarct heart compared with other treatment groups. A lowering of myocardial fibrosis of up to 60% by the phEPO/ABP polyplexes in the infarcted LV eventually suggests alleviate chamber stiffness, halting adverse cardiac remodeling.

3.4. phEPO/ABP preserves cardiomyocyte loss and lower apoptotic activity

The ongoing cardiomyocytes loss from necrosis or apoptosis is one of the early pathological characteristics in MI.[3, 29, 31] Thus, we analyzed the efficacies of the different treatments with regard to the loss of cardiomyocytes 10 days after MI (Fig. 4A and B). In the results of cTnT immunohistochemical staining, the adjusted percent of cardiomyocytes lost was significantly elevated in all of the treatment groups—rHuEPO, phEPO, phEPO/ABP, and phEPO/PEI—as with the I/R-only group ($P < 0.01$; Fig. 4B). Compared to the rHuEPO group, the phEPO/ABP ($P < 0.01$) and phEPO/PEI polyplex groups ($P < 0.05$) showed significantly decreased cardiomyocytes loss (Fig. 4A and B). Taken together, only the phEPO/ABP group showed significantly preserved cardiomyocyte numbers compared with other treatment groups ($P < 0.001$).

During post-infarct cardiac remodeling, reperfusion injury results in the paradoxical acceleration of apoptosis in the reperfused myocardium.[32] Then, we investigated the degree to which the administration of phEPO/ABP inhibits the apoptotic activity in the border zone of LV infarct (LVfb) were compared to other groups (Fig. 4C and D). The apoptotic activity measured by TUNEL staining revealed lower apoptosis in the phEPO/ABP polyplex injection group ($348.4 \pm 145.3/\text{mm}^2$) than that of other groups (Fig. 4D). Consistent with previous results, stronger inhibition of apoptosis in the phEPO/ABP treatment group diminished infarct size, favoring improvement in cardiac function after MI.

3.5. phEPO/ABP enhances angiogenesis and modulates the activation of myoFbs

During the healing phase of post-infarct cardiac remodeling, the blood supply to the infarcted myocardium is restored by angiogenesis and by remodeling of the vascular tree to conserve cardiac function [30, 31]. IHC staining for α -SMA showed more abundant arterioles in the phEPO/ABP polyplex injection group than in other treatment groups (Fig. 5A and B). The mean number of α -SMA-positive arterioles per hpf increased from 5.0 ± 0.6 in the I/R only group to 10.6 ± 1.0 in the phEPO/ABP polyplex injection group ($P < 0.01$; Fig. 5A and B). The administration of the phEPO/ABP polyplexes revealed a higher upregulation of angiogenic activity in the LVfb than other treated groups, which could

increase capillary-to-myocyte ratio, decrease the oxygen diffusion distance, and consequently improve oxygen supply to the infarcted myocardium.

During cardiac remodeling, the activated myofibroblasts (myoFb, collagen-secreting *de novo* α -SMA⁺-expressing fibroblasts) replace the lost cardiomyocytes and form nonregenerative scar tissue by depositing profibrotic molecules such as collagen and fibronectin in the extracellular matrix [31, 33, 34]. MyoFb is the predominant source of collagen mRNA in healing MI, which has the characteristics of fibroblasts and smooth muscle cells, has at least a twofold stronger contractile activity compared with α -SMA⁻ negative fibroblasts, and eventually determines the infarct size and quality of the scar.[6, 30, 33, 35] MyoFbs are present 4–6 days after an infarction and peak with maximum proliferation within the first two weeks after acute MI in humans.[35] To further elucidate the potent cardioprotective mechanism of phEPO/ABP, we examined the distribution and density of myoFbs by α -SMA expression in post-infarct cardiac remodeling between the different groups (Fig. 5C and D). There was comparable in α -SMA positivity for the phEPO-alone group and phEPO/PEI group compared with the I/R group (Fig. 5D).

The analysis of the adjusted α -SMA expression in the LVfb highlighted the distinct differences between the treatment groups (Fig. 5D). In particular, the rHuEPO group and the phEPO/ABP group represented two extremes of α -SMA activity in the LVfb. The phEPO/ABP group had up to a 75% decrease in α -SMA expression compared to that measured in the rHuEPO group and a 55% decrease compared to the I/R group ($P < 0.01$; Fig. 5D). The exaggerated activation of myoFbs after post-infarct cardiac remodeling is significantly modulated in the phEPO/ABP group compared with the other treatment groups. In the rHuEPO group, increased myoFbs may form fibrotic scars, preventing infarct expansion, ventricular dilation, and cardiac rupture. In addition, through their contractile activity, increased myoFbs generate tensile strength, helping the function of the infarcted heart. Collectively, the enhanced myoFb density in the extracellular matrix of the rHuEPO group contributes to the salutary effects of rHuEPO administration to compensate for postinfarct cardiac remodeling. However, the persistent and excessive activation of myoFbs in the rHuEPO group with the consequent collagen production causes deleterious cardiac remodeling and unfavorable outcomes, such as fibrosis, contracture, and heart failure. On the contrary, the phEPO/ABP group modulated the spread and abundance of myoFbs by controlling α -SMA-expressing myoFb differentiation, accompanied by the conservation of cardiomyocyte loss. This entirely different characteristic of the phEPO/ABP group may induce favorable anti-remodeling effects in the infarcted heart. From this viewpoint, we could weigh in on the analysis of myoFb infiltrations between the treatment groups.

3.6. phEPO/ABP suppresses pro-fibrotic Ang II effects on the heart

Neurohormones, such as Ang II and other inflammatory cytokines have functionally significant cross-talk, converging on common signal transduction pathways in cardiac remodeling after MI.[29, 36] Especially, the beneficial actions of the renin-angiotensin system (RAS) blockers making an impact upon patients survival are better correlated with the inhibition of tissue RAS levels rather than plasma levels [5, 6, 31]. Activation of the local cardiac tissue RAS, with its regulation independent of the systemic RAS, has important physiological and pathological roles, including post-infarct cardiac remodeling.[5] Beyond its regulation of blood pressure and fluid homeostasis, Ang II—the final physiologically active effector of RAS—has multiple effects on the heart, inducing myocyte apoptosis/necrosis and inflammation, driving perivascular fibrosis and scarring, stimulating fibroblast proliferation and collagen deposition, and inducing differentiation of cardiac fibroblasts into myoFbs.[6, 30, 34, 36] Blockers of RAS are clinically well-proven treatments in patients with MI, preventing LV remodeling and eventually improving survival.[5] Independent of their blood pressure-lowering effect, widely prescribed ACE-inhibitors and ARBs are able

to reverse the extent of myocardial fibrosis, reduce the LV chamber stiffness, and improve the LV function by pleiotropic and additional off-target effects on cardiac fibroblasts of the remodeling heart.[3, 5, 29, 34]

We next asked which underlying molecular mechanisms explain the potential effects of phEPO/ABP gene delivery, compared with other treatment groups. After I/R, Ang II expression increased in a whole subdivision of cardiac tissues ($P<0.05$; Fig. 6A and B). The suppression of Ang II expression in the phEPO/ABP group reached comparable levels to that of the thoracotomy group in the LVfb, RV, and atria, and it was at an even lower level than the thoracotomy group in the IVS (Fig. 6A and B). Compared with the I/R group, phEPO/ABP gene delivery showed remarkable decreases in Ang II in all of the cardiac tissues excluding the LVf ($P<0.05$; Fig. 6A and B). The phEPO/ABP group had significantly lower Ang II expression than that of the rHuEPO group in the LVfb, RV, and IVS ; than that of the phEPO group both in the atria and IVS ($P<0.05$; Fig. 6A and B). Collectively, compared with the rHuEPO and phEPO-alone group, the phEPO gene therapy delivered by the ABP polymer demonstrated a significant suppression of pro-inflammatory and profibrotic Ang II expression in the peri-infarct as well as at non-infarcted remote sites (IVS, RV, and atria), implying stronger and more far-reaching effects on post-infarct cardiac remodeling. However, in the LVf, all treatment groups failed to suppress Ang II expression.

3.7. phEPO/ABP reduces fibrogenic TGF- β activity on the heart

TGF- β is a major cytokine that both initiates and terminates tissue repair, and its sustained production underlies cardiac hypertrophy by interstitial fibrosis and phenotypic differentiation of cardiac fibroblasts to α -SMA⁺ myoFbs, causing the transition from an inflammatory to a proliferative phase during infarct healing.[29, 30, 33–35, 37] TGF- β 1 expression is upregulated in infarcted regions and in patients with fibrotic disorders.[30, 33, 34] Ang II directly stimulates TGF- β 1 production, thus initiating cardiac fibrosis during the transition from stable hypertrophy to heart failure with the upregulation of fibronectin and collagen genes, and blockade of the TGF- β signaling pathway results in significant amelioration of deleterious post-MI cardiac remodeling with down-regulation of the RAS [33, 34]. Here we analyzed the expression levels of TGF- β according to the anatomical division between the groups. TGF- β expressions were increased in all subdivisions of cardiac tissues after myocardial I/R ($P<0.05$; Figure 6C and D). Particularly in the IVS, the entire treatment group demonstrated a significant suppression of TGF- β expression compared with the I/R group ($P<0.05$; Figure 6D). The suppression of TGF- β expression in the phEPO/ABP group reached levels comparable to that of the thoracotomy group in the LVfb, RV, atria, and IVS, except for in the LVf (Figure 6C and D). This decreased expression of TGF- β in the phEPO/ABP group within the peri-infarct as well as the remote zones explains the complementary functional and histologic favorable anti-remodeling effects. These combined findings suggest that the phEPO/ABP group mitigates post-infarct cardiac fibrosis by preventing collagen-secreting α -SMA⁺ myoFb differentiation through the inhibition of Ang II and TGF- β .

Together with Ang II expression, measurement of TGF- β levels in cardiac anatomical subdivisions elucidated that EPO itself was insufficient to reverse the fibrosis-dominated disease process, like the LVf during cardiac remodeling. In addition, the relatively increased activity of Ang II and TGF- β in the rHuEPO and phEPO injection-alone groups accounts for a portion of the increased metabolic activity of the enhanced myoFbs. Therefore, the sustained release or expression of EPO modified by the delivery system—and not the short-acting administration of rHuEPO or phEPO—could be able to protect against the cardiac ischemic cascade at histological and molecular levels.

4. Discussion

In the present study, intramyocardial phEPO gene therapy delivered by the bioreducible ABP polymer demonstrates potential cardioprotective effects on post-infarct cardiac remodeling in rats, compared with the treatment of rHuEPO protein and naked phEPO plasmid-alone. The prominent effects of phEPO/ABP gene therapy are accompanied by the preservation of cardiac geometry and function, reduction in the density of fibrotic tissue, protection against cardiomyocyte loss, decrease in apoptotic activity, stimulation of angiogenesis, inhibition of α -SMA⁺ myoFb differentiation, and suppression of the profibrotic Ang II and TGF- β expression across the LVfb and remote non-infarcted sites in rats after MI.

We can speculate on the underlying mechanisms of the noteworthy cardioprotection from phEPO/ABP delivery after MI in detail. First, we previously reported that phEPO/ABP polyplexes protected *pDNA* from degradation *in vitro* for over 6 hours in the presence of serum, which allows for an increased circulation time *in vivo*. [22, 23] The effects of phEPO/ABP gene delivery on post-infarct cardiac remodeling are probably attributable to the characteristics of the bioreducible ABP carrier, inducing prolonged release and circulation times of the loaded phEPO gene. Otherwise, it is well known that the naked pDNA is not stable in blood and is degraded within minutes after intravenous injection. [22, 23] We assume that the too-short stability of phEPO itself in the blood was not enough to cover the cardiac remodeling process. Second, under clinical and anatomical backgrounds, the compact extracellular matrix of the myocardium filled with negatively charge molecules such as glycosaminoglycan and proteoglycan may be a major drawback for cardiac gene delivery, especially for positively charged particles, compared to neutralized and negatively charged naked plasmid DNA and siRNA-alone. This effect was apparent in the absence of efficacy displayed by the highly cationic PEI polyplex control group. Third, direct injection of pDNA itself into the cardiac muscle demonstrated 10–100 times more efficiency of gene expression than injection of the same amount of pDNA into skeletal muscle. [38] The treatment route of intramyocardial local injection may amplify the cardioprotective effect of the phEPO/ABP gene therapy. Fourth, inflammation is one of the main pathophysiologic mechanisms in postinfarct cardiac remodeling. [31] The *in vivo* innate immune response measured by the plasma IL-6 levels was comparable between the phEPO-alone and phEPO/ABP polyplex groups, even with a higher amount of phEPO and ratio of phEPO/ABP. [23] Fifth, the favorable pathologic findings of lessened fibrosis, and reduced necrosis in the phEPO/ABP polyplex group possibly allow the delivered phEPO gene to remain in the intact extracellular matrix of the post-infarcted heart to transfect viable cells. Sixth, the average size and distribution of the particles are important factors to determine the pharmacokinetics and pharmacodynamics of the delivered drug and gene. The phEPO/ABP polyplexes had a more condensed homogenous distribution than the phEPO/PEI polyplexes. Seventh, the superiority of the ABP polyplexes in cardiac remodeling may be explained by the well-known toxicity limitation of the PEI polymer, offsetting its positive biological effects. [20, 24]

5. Conclusions

The favorable cardioprotective effects of phEPO/ABP polyplexes are not confined only to the LV infarct lesion, as with ACE-Is or ARBs, and spread into non-infarcted sites including the IVS, RV, and atria. Functional, histopathologic, and molecular analysis between the well-known rHuEPO, phEPO-alone, and our phEPO/ABP delivery system has provided a deeper understanding of the subcellular remodeling process to help find an advanced therapeutic approach for MI. Unlike rHuEPO or phEPO treatment-alone, our phEPO gene therapy delivered by biodegradable ABP provides a promising tool for human gene therapy

to reverse post-infarct cardiac remodeling and eventually restore function to the same level as the thoracotomy control with supporting mechanisms. Thus, these intriguing reversal effects on cardiac remodeling by intramyocardial injections of phEPO/ABP polyplexes at the start of myocardial reperfusion suggest that this excellent bio-reducible delivery vector can revive the therapeutic potential of phEPO and other cardioprotective genes during post-infarct cardiac remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of phEPO/polymer polyplexes. Average particle size and Zeta potential of 50 µg phEPO/ABP polyplex (w/w=1:5) (A) and 50 µg phEPO/PEI polyplex (w/w=1:1) (B). Experimental time-dependent protocol (C).

Fig. 2. phEPO/ABP conserves cardiac geometry and function during post-infarct cardiac remodeling. The measurement of cardiac parameters by echocardiography 5 days after MI (A) and 10 days after MI (B), expressed as the mean $\pm$ SD (n=6–9 per group). The thickness of interventricular septum during systole (IVSs), thickness of interventricular septum during diastole (IVSd), left ventricular diameter during systole (LVDs), left ventricular diameter during diastole (LVDd); $^{*}P < 0.05$ vs thoracotomy, $^{\dagger}P < 0.05$ vs I/R group, $\#P < 0.05$ vs rHuEPO, $^{\S}P < 0.05$ vs phEPO-alone group, $^{\S}P < 0.05$ vs phEPO/PEI group.

Fig. 3.

Myocardial fibrosis by collagen contents and infarct size on post-infarct days 10 after intramyocardial administration. (A) Representative Masson's trichrome staining images in the mid-ventricle of hearts from each group. Bar=2 mm. (B) Quantification of percent fibrosis area in LV. (mean ± SD; n = 4–6 per group). * $P < 0.01$ vs I/R group, # $P < 0.01$ vs rHuEPO, † $P < 0.01$ vs phEPO-alone group, § $P < 0.01$ vs phEPO/PEI group.

Fig. 4.

Cardiomyocytes loss and apoptotic activity 10 days after MI. (A) Representative IHC staining images for cTnT in the mid-ventricle of hearts from each group (n=4–6). Bar=2 mm. (B) Quantification of percent cardiomyocytes loss in LV adjusted by the level of thoracotomy group (mean±SD; n=4–6 per group). (C) Representative TUNEL staining images in the LVfb from each group. Bar=200 µm. (D) Quantification of corrected TUNEL positive cells (mm²) corrected by the level of thoracotomy group (mean±SEM; n=4–6 per group). * $P < 0.01$ vs I/R group, # $P < 0.05$ vs rHuEPO, ## $P < 0.01$ vs rHuEPO, † $P < 0.01$ vs phEPO-alone group, § $P < 0.01$ vs phEPO/PEI group.

Fig. 5.

Angiogenesis (A, B) and modulation of myoFbs differentiation (C, D) in the LVfb according to different treatments 10 days after MI. (A) Representative IHC staining images of the α -SMA-positive arterioles. Bar=200 μ m. (B) Quantification of pro-angiogenic activity by the α -SMA-positive arterioles adjusted by the level of thoracotomy group. (C) Ventricular myoFbs differentiation is analyzed by measuring α -SMA expression as a marker of myoFb. Representative interstitial IHC staining images of α -SMA-positive myoFb infiltrations. Bars=100 μ m. (D) Quantification of α -SMA-positive myoFb differentiation adjusted by the level of thoracotomy group (mean $\pm$ SD; n=4–6 per group). * P <0.01 vs I/R, # P <0.01 vs rHuEPO, † P <0.01 vs phEPO-alone group, § P <0.01 vs phEPO/PEI group.

Fig. 6. Fibrogenic Ang II (A, B) and TGF-β (C, D) expression between different treatment groups in the subdivision of cardiac tissues – LVinfarct (A, C) and non-infarcted remote sites (B, D). Representative image of Western blots and densitometric quantitative analysis normalized to anti-Actin (β-sarcomeric) expression in rat hearts (mean±SEM; n=4–7 per group). ABP, 50 μg phEPO/ABP (w/w=1:5); PEI, 50 μg phEPO/PEI (w/w=1:1); ‡ $P < 0.05$ vs thoracotomy, * $P < 0.05$ vs I/R group, # $P < 0.05$ vs rHuEPO, † $P < 0.05$ vs phEPO-alone group, § $P < 0.05$ vs phEPO/PEI group.