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Functional Polymers of Gene Delivery for Treatment of Myocardial Infarct

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Abstract

Ischemic heart disease is rapidly growing as the common cause of death in the world. It is a disease that occurs as a result of coronary artery stenosis and is caused by the lack of oxygen within cardiac muscles due to an imbalance between oxygen supply and demand. The conventional medical therapy is focused on the use of drug eluting stents, coronary-artery bypass graft surgery and anti-thrombosis. Gene therapy provides great opportunities for treatment of cardiovascular disease. In order for gene therapy to be successful, the development of proper gene delivery systems and hypoxia-regulated gene expression vectors are the most important factors. Several non-viral gene transfer methods have been developed to overcome the safety problems of viral transduction. Some of which include plasmids that regulate gene expression that is controlled by environment specific promoters in the transcriptional or the translational level. This review explores polymeric gene carriers that target the myocardium and hypoxia-inducible vectors, which regulate gene expression in response to hypoxia, and their application in animal myocardial infarction models.

Keywords

Myocardial infarct; Gene delivery; Non-viral carrier

1. Introduction

Myocardial infarction (MI) is the leading cause of death in developed nations and one of the most common causes of death in the world. The blockage in coronary arteries by atherosclerosis or thrombus develops ischemic heart disease that includes temporary pain (angina), irregular heart beat (arrhythmia), permanent heart muscle damage (MI), and loss of

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muscle activity (heart failure) [1]. Cardiac remodeling leading to heart failure is a global and cellular change in ventricular shape and function following chamber dilation, interstitial and perivascular fibrosis. This includes neurohormonal responses, cytokine activation, loss of cardiomyocytes due to necrosis or apoptosis, cardiomyocyte hypertrophy, disruption of extracellular matrix (ECM) and collagen accumulation followed by scar formation [2]. Unfortunately, current pharmacological treatment regimens for myocardial infarction do not reliably limit remodeling of the left ventricle (LV) post-infarction and prevent progression to heart failure [3]. Novel potential treatments, including gene and cell therapies, offer a means to directly treat the pathophysiology underlying the long-term complications of myocardial infarction-loss of cardiomyocytes. The process of remodeling of the left ventricle begins immediately after an acute ischemic insult. The extent of remodeling correlates with the size of the infarct and the decline in cardiac function [4]. Oxidative stress resulting from rapid metabolic changes in the early stages of ischemia plays a crucial role in cardiomyocyte apoptosis and fibrosis of the myocardium [5]. The extent of cardiomyocyte loss in the early stages following an acute MI correlates directly with the subsequent degree of left ventricular remodeling and the decline in cardiac function. This suggests that preventing the loss of cardiomyocytes in the early stages of an acute MI is necessary to achieve long-term efficacy in the treatment of ischemic heart disease.

Since it was first reported in 1972, gene therapy has been a rapidly progressing technology for treating many genetic and acquired diseases including myocardial infarction [6]. The genetic intervention includes (1) overexpression of a target molecule by the introduction of plasmid DNA, (2) a loss-of-function approach by the introduction of RNA interference (RNAi), and (3) correcting deleterious gene mutations/deletions at the genome or primary mRNA level. Neovascularization and the inhibition of apoptosis are considered as good approaches for the sequentially combined gene therapy for ischemic disease. In the early stage of myocardial infarct, reduced oxygen supply and increased reactive oxygen species (ROS) occur in ischemic cardiomyocytes followed by apoptosis. Protecting the cells from apoptosis is the first step, and the second step is to reestablish vasculature through angiogenesis that returns the hypoxic condition back to a normoxic state. DNA, small interfering RNA (siRNA), and micro RNA have been applied to gene therapy. DNA-based gene therapy delivers exogenous plasmid DNA to the cellular nucleus, which encodes a specific gene that enhances the expression of therapeutic proteins. On the other hand, siRNA reduces protein expression by silencing target mRNA in the cellular cytoplasm. However, they must overcome several barriers for successful clinical application such as cell membrane penetration, stability in serum, and safety concerns such as un-controlled gene delivery [7]. To overcome those barriers, DNA and RNA require appropriate delivery vehicles. Various non-viral carriers such as cationic polymers, peptides, liposomes and nanoparticles have been developed and have showed success in the delivery of genes through the cell membrane and into the cell, thus protecting genes from degradation [8].

In 1997, with rationales including a versatile design, no integration into the host chromosome, and non-immunogenic response, research regarding polymeric gene delivery was started [9]. Polymeric carriers are safe for repeated injection, easy to reproduce, and cost-effective, all of which are fundamental considerations in the development of pharmaceutical products [8]. Despite the benefits, they typically show relatively low

transfection efficiency and poor therapeutic efficacy compared to virus-mediated gene delivery [10]. Various polymer constructions have been developed to overcome the drawback of polymeric gene carriers. In this review, we describe the use of polymeric gene carriers for treatment of myocardial infarction.

2. Cardiovascular Gene Therapy

A variety of catheter or surgical approaches for in vivo gene transfer into myocardial tissue showed promising results in animal and clinical studies. Specifically, angiogenic gene therapy is of growing interest as an alternative treatment to the conventional protein therapy in the area of ischemic heart diseases. An animal model with chronic ischemic myocardium showed an increase in collateral blood flow and an improvement of cardiac function by the injection of plasmid vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) [11, 12]. In human clinical trials, the administration of plasmid VEGF into ischemic myocardium through a left anterior thoracotomy resulted in improved heart responses [13]. Similar to VEGF, administration of FGF into epicardial fat also demonstrated an improvement of angina symptoms and an increase in myocardial blood flow [14]. In addition, anti-apoptotic genes and anti-oxidative genes have been widely used in cardiovascular gene therapy. Despite its great potential as treatment to ischemic heart diseases, the transfection efficiency, stability, safety and controlled expression of the therapeutic genes need to be assured.

3. Polymeric Gene Carriers

A variety of polymer-based gene delivery systems have been developed in the last decade to improve efficacy of therapeutic genes. The polymeric carriers are typically positively charged to bind with the negatively charged the cell membrane that blocks gene transfer into cells [7]. Cationic polymers readily condense negatively charged nucleic acids through electrostatic interaction and have thus been widely used as gene carriers [15-17]. The binding affinity between carriers and nucleic acids may decrease the expression of genes because nucleic acids have to be dissociated from the carrier to move to their target location inside the cells [18]. Designing bioreducible polymers with a proton buffering effect for endosomal escape and rapid dissociation in cytoplasm has solved this problem [19]. Bioreducible polymers, or peptides containing internal disulfide bonds in the main chain, at the side chain or in the cross-linker, have high stabilities in extracellular spaces and are rapidly reduced in the cytosol by high intracellular glutathione (GSH) [20]. Polymer reduction can reduce the cytotoxicity of high molecular weight polycations by converting the polymers back into the smaller constitutive subunits and also allow for the release of nucleic acids into the cytoplasm [21]. The use of bioreducible polymers in gene therapy is increasing due to their potential for enhanced transfection efficiency and cytoplasm-sensitive gene delivery.

4. TerplexDNA

A gene delivery carrier was developed that is derived from stearyl-poly(L-lysine) (stearyl-PLL), low density lipoprotein (LDL) and plasmid DNA. TerplexDNA is generated through electrostatic and hydrophobic interactions between LDL, stearyl-PLL, and DNA [22, 23].

The PLL component condenses DNA through the interaction of the epsilon-amino group of the lysine with the negatively charged phosphate backbone of DNA [22, 23]. The stearyl groups participate in hydrophobic interactions with the core of the LDL molecule allowing integration into the molecule itself [24]. Incorporation of LDL into the polymer gene carrier enhances gene delivery through augmentation of the LDL receptor-mediated endocytosis pathway. LDL receptors exist on the surface of many cells including artery endothelial cells, myocytes, and hepatocytes [25, 26].

Compared with Lipofectamine, the TerplexDNA system showed high transfection efficiency without cytotoxicity in a human hepatocyte line (HepG2), murine smooth muscle (A7R5) cell lines [22, 23], and bovine aorta primary cell cultures, both vascular smooth muscle cells and endothelial cells [24]. Pharmacokinetic and biodistribution studies revealed that TerplexDNA improved circulation time and prolonged whole body retention. TeplexDNA was less toxic than other cationic polymers because the mechanism of internalization is receptor-mediated endocytosis [23, 27]. The main advantage of TerplexDNA might be due in part to the prolonged half-life, which provides the benefit of an increased chance of interaction with the LDL receptor. Another advantage might be the possibility of repeated administration because TerplexDNA is non-immunologic [23, 27]. The TerplexDNA system may therefore have applications in the treatment of heart disease in a clinical setting.

4.1 Gene Delivery into Myocardium

The TerplexDNA system would be beneficial for delivering DNA to the myocardium because the LDL receptors exist on the surface of myocytes [28]. The transfection efficiency of TerplexDNA in rabbit myocardium was 20-100-fold higher than that of naked DNA. This system exhibited more widespread and uniform gene expression near the injection area. In a rat myocardial infarction model, gene transfection was significantly improved without toxicity when compared to naked DNA. TerplexDNA system was developed as an efficient gene carrier that has potential in future clinical applications for the treatment of cardiovascular diseases.

4.2 Gene Delivery into Primary Artery Wall Cells

Receptor-mediated endocytosis was found to be the main mechanism of TerplexDNA internalization into cells [22, 23]. Along with the fact that the cells of the artery wall also express LDL receptors on their cell surface [25], it was hypothesized that TerplexDNA system specifically delivers genes into these cells. This TerplexDNA system was evaluated with these cells for transfecting reporter genes (β-galactosidase and luciferase) or VEGF gene. TerplexDNA system specifically delivered reporter genes and VEGF gene into the cells of the bovine aortic artery wall by receptor mediated endocytosis [24]. The gene transfection by TerplexDNA system was significantly inhibited in the presence of free LDL. This proves that TerplexDNA system is a promising tool for artery wall gene transfer.

4.3 VEGF Gene Delivery in Animal Model

The mechanism of left ventricular dysfunction after myocardial infarction is multifactorial and not completely understood [29]. It was reported that the essential role of myocytes that secrete VEGF was to maintain the function of the left ventricle [30]. This finding suggests

the autocrine and paracrine role of VEGF in the maintenance of normal function of the myocardium and the left ventricular extracellular matrix, which can be noted as an important factor to prevent the progress of myocardial infarction. The Protective effects of VEGF on the left ventricular function were proved in an ex vivo model of acute ischemia [31]. Positive therapeutic efficacy was observed from an animal model that was administered TerplexDNA system delivering plasmid VEGF [32]. The improvement in left ventricular function in the VEGF-treated animals was accompanied by an inhibition of the usual increase in left ventricular systolic and diastolic area as seen commonly following a myocardial infarction.

5. Water Soluble Lipopolymer (WSLP)

WSLP was synthesized by combining the cationic head group of branched PEI (bPEI 1.8 KDa) with a hydrophobic lipid anchor, cholesterol chloroformate. It was reported as an efficient gene delivery carrier [33-35]. High molecular weight branched PEI (bPEI 25 kDa) has been known as a cationic polymer that has shown high transfection efficiency due to the proton buffering effect. However, bPEI25 kDa is highly toxic to cells [36]. Low molecular weight bPEI such as bPEI1800 is less cytotoxic, but shows low efficiency in gene transfection. WSLP has many advantages over PEIs. First, WSLP contains the PEI moiety, which has the proton buffering effect. This buffering effect facilitates endosome escape, resulting in high transfection efficiency. Second, WSLP is less toxic to cells because it contains bPEI1800. Third, the cholesterol moiety in WSLP enhances transfection efficiency. Cholesterol is taken up by the cells through receptor-mediated endocytosis or transfer from the lipoprotein to the exoplasmic leaflet of the plasma membrane bilayer [37]. Fourth, WSLP does not require a co-lipid, unlike other cholesterol-based cationic lipids. Since WSLP can escape the endosome, other co-lipids that destabilize the endosomal membrane are not necessary [33, 34, 38].

5.1 Gene Delivery into Myocardium

WSLP was evaluated as a gene carrier to smooth muscle cells in vitro and to the myocardium in vivo [34]. The transfection efficiency of WSLP was higher than that of PEI1800, lipofectamine, or SuperFect to A7R5 smooth muscle cells. In addition, WSLP showed negligible cytotoxicity to A7R5 cells. The uptake mechanism studies suggested that WSLP internalized into cells through a cholesterol-mediated uptake mechanism [34]. Moreover, the injection of WSLP/pDNA to rabbit myocardium also showed that WSLP had higher transfection efficiency than PEI1800.

5.2 VEGF Gene Delivery in Animal Model

The construction of an effective plasmid DNA that encodes a specific therapeutic gene is important for successful gene therapy. VEGF has been known to be the most effective therapeutic protein for the neovascularization [39]. However, Springer et al. proved that exogenously delivered VEGF could exert a physiological effect in normal, nonischemic tissues [40]. This suggested that VEGF expression must be carefully modulated. In addition, unregulated continuous expression of VEGF is associated with the formation of endothelial cell-derived intramural vascular tumors [41].

Erythropoietin (Epo) enhancer was previously reported to enhance the activity of the promoter under hypoxia conditions [42-44]. Those studies showed that the Epo enhancer induced reporter gene expression in various cell lines under hypoxia, suggesting that the Epo enhancer could regulate gene expression. Therefore, the Epo enhancer is a good gene regulator to express VEGF in response to hypoxia. A hypoxia-inducible plasmid expressing VEGF was constructed by adding the Epo enhancer in the upstream of the VEGF coding region [35]. The efficiency of the pEpo-SV-VEGF/WSLP complex was evaluated in vitro and in vivo. The promoter has a hypoxia-responsive element that binds to hypoxia-inducible factor-1 (HIF-1) and activates the transcription of the gene under hypoxia conditions [45-47]. After transcription, the VEGF mRNA is stabilized by cooperation of multiple RNA elements such as coding regions and 5′- and 3′-untranslated regions (UTRs), resulting in an increase of the translation rate [48]. Several hypoxia-regulated genes including Epo have hypoxia-responsive elements in their promoters and enhancers. By employing the Epo enhancer for the regulation of the VEGF gene, we mimicked the natural regulation of VEGF expression. Hypoxia-regulated gene expression in the mouse ischemic heart model was reported using a hypoxiaresponsive element by adeno-associated virus-mediated gene transfer [49]. In addition, the Epo-enhancer-mediated induction of the VEGF gene was proven in a rabbit ischemic heart model. These results suggested that the hypoxia-inducible VEGF gene delivery system in combination with WSLP is effective and safe for the treatment of ischemic heart disease.

Springer et al. proved that exogenously delivered VEGF could exert an unknown effect in normal and non-ischemic tissues [40]. In addition, unregulated continuous expression of VEGF is associated with formation of endothelial cell–derived intramural vascular tumors [41]. These findings suggest that VEGF expression must be regulated. It has been shown that Epo enhancer-SV40 promoter induced gene expression in vitro under hypoxia and in rabbit ischemic myocardium in vivo [35]. In addition, Su et al. proved the over-expression of the hypoxia-responsive element-induced VEGF in ischemic myocardium by using an adeno-associated virus [49]. Hypoxia-specific regulation of the VEGF expression system may be useful for safer VEGF gene therapy by minimizing unwanted side effects.

6. Ischemic Myocardium-Specific Gene Regulation Systems

6.1 RTP801 Promoter

The RTP801 promoter is an effective regulatory system in response to hypoxia. Shoshani et al. reported that RTP801 transcription was rapidly and sharply increased both in vitro and in vivo under hypoxic condition [50]. This inducible expression of RTP801 is mediated by transcriptional activation. In addition, it was suggested that the induction of the RTP801 promoter was mediated by HIF-1, which is a transcription factor that mediates hypoxia induction of a number of genes [51, 52]. Binding of HIF-1 to the consensus domain of the genes results in the transcriptional induction of the gene promoters [43, 53]. HIF-1–mediated induction of gene transcription is a widespread oxygen-sensing mechanism in various types of cells [44].

The RTP801 promoter was analyzed and identified a cis-regulatory element that was responsible for the hypoxia induction of the promoter [54]. A potential Sp1 element in the

RTP801 promoter was involved in hypoxia induction. It was shown that the RTP801 promoter was induced under hypoxia conditions in various types of cells. A VEGF plasmid including the RTP801 promoter controlled VEGF expression under hypoxia. This promoter could minimize the side effect of the nonspecific VEGF expression and induce VEGF expression specifically in ischemic tissue. The results suggested that a hypoxia-inducible VEGF plasmid, pRTP801-VEGF, might be a useful angiogenic gene for the treatment of ischemic diseases.

In another study, delivery of RTP-VEGF plasmid using a novel reducible disulfide poly(amido ethylenediamine) (SS-PAED) polymer carrier was studied in vitro and in vivo [55]. In vitro transfection into rat cardiomyocytes (H9C2) showed that SS-PAED increased the gene expression by 16-fold compared to bPEI control. SS-PAED mediated delivery of RTP-VEGF plasmid produced significantly higher levels of VEGF expression (up to 76 fold) under hypoxic conditions compared to normoxic conditions in both H9C2 and rat aortic smooth muscle cells (A7R5). Using SS-PAED, delivery of RTP-VEGF was investigated in a rabbit myocardial infarct model. Results showed up to 4-fold increase in VEGF protein expression in the region of the infarct compared to injections of SS-PAED/RTP-Luc.

Although unregulated, constitutively expressed VEGF gene therapy reduced myocardial infarct size, ischemia-inducible VEGF gene therapy demonstrated even greater efficacy. The mechanism for the improved efficacy seen with the ischemia-inducible VEGF gene therapy is as follows: (1) decreased apoptosis and (2) increased angiogenesis, compared with unregulated, constitutively expressed VEGF gene therapy following myocardial infarction. It is likely that both mechanisms contribute to the significant decrease in myocardial infarct size. In addition to these two mechanisms, VEGF gene therapy has previously been shown to (1) promote arteriogenesis and (2) have a mitogenic effect on adult cardiomyocytes in large animal models of myocardial ischemia and infarction [56-58]. Both of these previously described mechanisms most likely also contribute to the significant decrease in myocardial infarct size.

6.2 Epo Enhancer

The activity of the Epo enhancer-SV40 promoter system was further enhanced without significant decrease in its specificity by co-transfection of HIF-1-alpha gene [59]. Co-transfection of pSV-HIF1 alpha and pEpo-SV-Luc increased the promoter activity of the Epo enhancer-SV40 promoter system, showing at least a 3-fold higher gene expression under hypoxia as compared with the pEpo-SV-Luc single-plasmid transfection. Furthermore, co-transfection showed significant hypoxia specificity. Also, co-transfection of pEpo-SV-VEGF with pSV-HIF-1-alpha showed enhanced VEGF expression without loss of hypoxia specificity when compared with pEpo-SV-VEGF single-plasmid transfection. Furthermore, pSV-HIF-1-alpha induced the endogenous hypoxia-responsive genes such as angiopoietin-1, which would be beneficial for therapeutic angiogenesis. Therefore, with hypoxia specificity and higher gene expression, co-transfection of pSV-HIF-1-alpha and pEpo-SV-VEGF may be useful for ischemia targeting gene therapy.

6.3 Post-translational Gene Regulation System

Hypoxia-responsible gene expression can be primarily regulated in three ways: 1) transcriptional regulation; 2) post-transcriptional regulation, and 3) post-translational regulation [60]. HIF-1 is accepted as the most important element in transcriptional regulation. In post-transcriptional regulation, VEGF mRNA is stabilized under hypoxic conditions by the cooperation of the 5′- and 3′-untranslated regions (UTRs) and the coding region. The best-studied strategy to control gene expression under hypoxic conditions in post-translational regulation is the fusion of the oxygen-dependent degradation (ODD) domain and the therapeutic gene in order to stabilize the protein produced [61, 62]. The use of the ODD domain constitutes a powerful tool to increase the resident time of proteins, which typically have a short half-life. VEGF in particular needs to be stabilized following expression because its half-life is very short [63-65]. Unlike the RTP801, the pβ-SP-ODD-VEGF composed of the signal peptide, the ODD domain, and the furin-cleavage site regulates VEGF secretion in response to hypoxic conditions at the post-translational level [66]. The ODD domain stabilizes VEGF in hypoxic cells, while the furin site is cleaved by furin enzyme in the Golgi apparatus, leading to the production of wild-type VEGF. The SP domain then facilitates the exogenous secretion of this wild-type VEGF, which is known as the most potent form of VEGF [67, 68].

While the presence of the ODD domain can play a positive role in post-translational regulation, its large molecular weight may cause an abnormal folding or a decrease in secretion of the therapeutic protein, thereby diminishing the efficacy of the secreted therapeutic protein [60, 69]. These potential shortcomings may be overcome by the use of a short ODD domain composed of 18 core amino acids [69]. However, this shorter ODD domain may interfere with the interaction between VEGF and its receptors. Enzymatic degradation between the ODD domain and VEGF helps to ensure that the wild-type form of VEGF is secreted following processing in the Golgi network [70].

Furin, a member of the subtilisin-like proprotein convertase family that exists in the Trans Golgi Network (TGN), processes latent precursor proteins into their biologically active products in the secretory pathway [71]. Furin recognizes a conserved polybasic R-X-R/K-R site and cleaves that site downstream of the target sequence [72, 73]. R-G-R-R, a furin-recognition site, was inserted between the ODD domain and the VEGF-coding region to enhance the secretion of wild type VEGF [74]. The decrease in VEGF secretion seen with the increase in the concentration of the furin inhibitor suggests that VEGF secretion is accelerated by the cleavage of the signal peptide (SP) and the ODD domains. The finding that the furin inhibitor hinders VEGF secretion and increases the intra-cellular concentration of VEGF also confirms that the furin-recognition site is a powerful tool to enhance the secretion of wild-type VEGF.

A plasmid containing the SP domain, the ODD domain, and the furin-cleavage site is a promising construct for enhancing VEGF secretion and the activity of VEGF under hypoxic conditions [66]. The SP domain directs VEGF into the secretory pathway while the ODD domain stabilizes the expression of VEGF. The furin-recognition site provides a specific region to separate VEGF from the SP and ODD domains, resulting in more efficient secretion of wild-type VEGF. This pβ-SP-ODD-VEGF plasmid was more efficient to

produce wild-type VEGF than the pRPT-VEGF plasmid, demonstrating that this plasmid construct is superior to other hypoxia-inducible VEGF plasmids. These findings suggest that pβ-SP-ODD-VEGF may be a promising gene construct for the treatment of a variety of clinically important ischemic disease states.

The protective effects of VEGF gene therapy in the setting of acute myocardial ischemia are due not only to the induction of angiogenesis but also to the prevention of apoptosis of the cardiomyocytes. The extent of the inhibition of apoptosis is directly correlated with the amount of VEGF released [75, 76]. The significant reduction in the number of apoptotic cardiomyocytes and the size of the myocardial infarcts in the dendrimer type-arginine-grafted bioreducible poly(CBA-DAH) (ABP) PAM-ABP/pβ-SP-ODD-VEGF treated hearts demonstrate that the pβ-SP-ODD-VEGF is more efficacious at preventing left ventricular remodeling and preserving left ventricular function than the RTP-VEGF. This is consistent with previously published VEGF secretion data [66] showing more efficient secretion of wild-type VEGF using the pβ-SP-ODD-VEGF plasmid compared to the RTP-VEGF plasmid.

The difference in efficacy between the RTP-VEGF and the pβ-SP-ODD-VEGF is, at least in part, due to where the hypoxia-responsive regulation of gene expression occurs. The RTP801 promoter includes HIF-1 binding sites and stimulating protein-1 (Sp1) that is up regulated under hypoxic conditions. Formation of a multi-protein complex of Sp1 with HIF-1 and Smad3, used as a co-activator and adaptor protein, results in an Sp1-Smad3-HIF-1 complex on the RTP801 promoter that works to enhance gene expression under hypoxic conditions [60, 77]. This hypoxia-responsive regulation of VEGF expression and secretion is the mechanism underlying the differences in the therapeutic effects of the pβ-SP-ODD-VEGF and the RTP-VEGF observed in previous studies [78]. It has been demonstrated that hypoxia-responsive VEGF gene therapy improves left ventricular function and prevents left ventricular remodeling following acute myocardial ischemia by inhibiting apoptosis of cardiomyocytes and promoting angiogenesis. When comparing two different hypoxia-responsive regulation systems, i.e. transcriptional versus post-translational regulation, pβ-SP-ODD-VEGF, the post-translationally regulated hypoxia-responsive plasmid was more efficacious than RTP-VEGF, a transcriptionally regulated hypoxia-inducible system, at improving the effects of ischemia/reperfusion injury in a rat model. The PAM-ABP/pβ-SP-ODD-VEGF shows promise as a potential novel therapy for the treatment of myocardial ischemia and infarction.

6.4 Combined Gene Delivery

Single gene therapy has failed to prevent the lethal arrhythmias, acute cardiogenic shock, and chronic end-stage heart failure, which are the potentially fatal complications of ischemic heart disease [79]. Sequentially combined gene therapy is a promising treatment for ischemic diseases because the single gene therapies are effective for either preventing apoptosis of ischemic cells or inducing angiogenesis. Gene therapy for ischemic diseases requires the induction of neovascularization and the inhibition of apoptosis. In addition, successful ischemic disease gene therapy via the above approaches needs sufficient genetic interventions based on a precise basic understanding of the mechanisms of heart failure.

A hypoxia-inducible plasmid was constructed for the dual expression of heme oxygenase-1 (HO-1; knock-in, anti-oxidation) and the Src homology domain-2 containing tyrosine phosphatase-1 (SHP-1) microRNA (miSHP-1; knockdown, anti-apoptosis). In addition to the anti-oxidative and the anti-apoptotic effects of HO-1 and mi SHP-1, several studies have reported that the overexpression of HO-1 and the silencing of SHP-1 accelerate angiogenesis in ischemic myocardium [80-82]. HO-1, a stress-inducible antioxidant enzyme, exerts potent cardioprotective effects through its anti-inflammatory, anti-apoptotic, and anti-oxidant activity in ischemic tissue [83]. HO-1 gene therapy may protect the heart from ischemia/reperfusion injury by suppressing the early inflammatory response and inhibiting cardiomyocyte apoptosis [84].

SHP-1, a key molecular mediator of apoptosis, negatively regulates anti-apoptotic signaling pathways, including extracellular signal-regulated kinase (ERK1/2) and BCL-2. SHP-1 binding to death receptors such as TNFR-1 and FAS-R promotes apoptosis through the regulation of de-phosphorylation in signal transduction pathways [85, 86]. Decreased cardiomyocyte apoptosis and increased cardio-protection through Akt activation by the inhibition of the SHP-1 gene suggest that a therapeutic strategy designed to inhibit the expression of SHP-1 by miRNA would be effective in IHD [87, 88]. Although miSHP-1 can reduce apoptosis and protect cells in ischemic tissue by inhibiting SHP-1 gene expression, induction of elevated levels of RNAi may be toxic to cells due to the interference with intrinsic cellular RNAi processes. Unlike siRNA, miRNA production can be regulated by promoters/enhancers, which transcribe genes in response to specific intracellular environments or signals, ensuring that RNAi activity occurs in specific tissues or cell conditions [89].

The hypoxia-inducible plasmid for the dual expression of HO-1 and miSHP-1 demonstrated higher levels of HO-1 expression and mature miSHP-1 production in hypoxic cells compared to normoxic cells [90]. In addition, both HO-1 and miSHP-1 can synergistically enhance the secretion of VEGF. Several studies have reported that both HO-1 overexpression and SHP-1 silencing accelerate angiogenesis in ischemic areas [80-82]. The combined gene delivery demonstrated in these studies is promising for ischemic diseases in terms of not only synergistic efficacy but also sequential activities that prevent cardiomyocyte apoptosis and induce angiogenesis [91] because antiapoptotic genes typically protect cells in ischemic tissues for up to 2 weeks, and neovascularization requires more than 1 week upon VEGF secretion [92]. This sequentially and synergistically combined gene therapy provides the double effects of cardiomyocyte protection in the early stage of ischemia and vascular regeneration in the late stage.

7. Targeted Gene Delivery into Myocardium

7.1 Artery Wall-Targeted Gene Delivery

Apolipoprotein B-100 (apo B-100), a major protein component of LDL, contains many receptor-binding domains such as LDL receptor-binding domain, artery wall cell-binding domain, and heparin-binding domain. Shih et al. demonstrated that a synthetic peptide containing 1000–1016 amino acid residues of apo B-100 (Arg-Ala-Leu-Val-Asp-Thr-Leu-Lys-Phe-Val-Thr-Gln-Ala-Glu-Gly-Ala-Lys) was the arterial wall-binding domain of apo

B-100 [26]. The focal accumulation of 125I-labled apo B-based synthetic peptide at the healing edges of regenerating endothelial islands in balloon-cather deendothelialized rabbit aorta suggested that this arterial wall-binding peptide could mediate accumulation of LDLs in arterial lesions. A synthetic peptide was selected based on apo B-100 and added cysteine at the N-terminus of the peptide to facilitate conjugation to PEG-g-PLL [93]. An artery wall-targeted gene delivery system based on PEG-g-PLL was synthesized by introducing the artery wall binding peptide (AWBP) to the end of PEG-g-PLL. The AWBP provided a means of targeting to the arterial wall cells and was shown to locate at the target site for a sufficient amount of time to treat the genetic problem. Luciferase gene transfer using AWBP-PEG-PLL confirmed that the targeted gene delivery to bovine aorta wall cells was mediated by specific artery wall cell receptor-mediated endocytosis.

7.2 Prostaglandin E2 (PGE2) targeting

Specific ligand-modified gene delivery systems are promising strategies to successfully control gene expression in target cells. The specific ligands bind to cell surface receptors and increase cellular uptake of therapeutic genes via target receptor-mediated endocytosis [94]. There have been many cell-selective siRNA delivery systems constructed using specific ligands for cancer or liver cells [95, 96]. Cardiovascular targets for siRNA therapy, however, have no specific vector system, except physical targeting methods. PGE2 is involved in numerous physiological mechanisms including the contraction and relaxation of smooth muscle, control of blood pressure, and modulation of inflammation [97]. Its diverse physiologic effects are exerted via four distinct PGE2 receptors (EP1-4) located on the cell surface [98], and PGE2 receptors (EPs) participate in agonist-induced internalization upon PGE2 stimulation [99]. Although the expression levels of EPs vary between different species, EP4 has been reported to be highly expressed in the hearts of several species, including humans [100]. Accordingly, it was hypothesized that the use of PGE2 as a specific ligand for cardiovascular-targeted siRNA delivery would increase the efficiency of siRNA transfer to cardiomyocytes.

Linear and branched types of reducible cationic copolymers (disulfide-containing poly(amido amine)s, SS-PAAs), synthesized by Michael-type addition between various amine-containing monomers and cystamine bis-acrylamide, have been reported as effective gene carriers [55, 101-103]. A linear SS-PAA, synthesized by copolymerization between 1,6-diaminohexane and cystamine bis-acrylamide (cystamine bisacrylamide-diamino hexane (poly(DAH/CBA)), served as a powerful vector to carry Fas siRNA into rat cardiomyocytes [104]. PGE2 was conjugated to Fas siRNA molecules, and the synthesized PGE2–Fas siRNA contributed to cardiomyocyte targeting via PGE2 receptor-mediated intracellular delivery. Cardiomyocyte apoptosis is the leading cause of heart failure after myocardial infarction. It has been reported that many apoptosis-related genes including Fas/Fas ligand are overexpressed in apoptotic cardiomyocytes. For successful in vivo application of siRNA therapeutics, it is necessary to develop a cardiomyocyte-targeted siRNA delivery system with high transfection efficiency. These results suggest that cardiomyocyte-targeted Fas siRNA delivery using the PGE2–Fas siRNA/poly(DAH/CBA) polyplex is a promising approach to inhibit apoptosis in the treatment of cardiovascular disease.

7.3 Primary Cardiomyocyte (PCM) Targeting

PCM is an isolated phage that displays a 20 amino acid peptide (WLSEAGPVVTVRALRGTGSW), which binds to primary cardiomyocytes 180 times more avidly than control phages [105, 106]. The PCM peptide sequence was used as a specific cardiomyocyte-targeting ligand for the delivery of genes and the potential treatment of cardiovascular disease. The Fas has been identified as an inducer of cardiomyocyte apoptosis, and many studies using Fas siRNA have demonstrated that inhibition of Fas, in turn hinders cardiomyocyte apoptosis without immune stimulation [107-109]. The ability of a synthesized PCM-modified bioreducible polymer containing Fas siRNA to down-regulate Fas gene expression and inhibit cardiomyocyte apoptosis has been described previously [110]. The study was designed to deliver siRNA effectively to a specific target, cardiomyocytes, with a high transfection efficiency, by modifying siRNA with PCM or cell-penetrating (Tat) peptides [111]. These peptide-conjugated siRNAs did not initially produce dense nanoparticles with CBA-DAH, but the addition of plasmid DNA resulted in a more stable and compact polyplex formation. The resulting compact C-siRNA-pDNA/CD polyplexes promoted high levels of cellular uptake and effective gene silencing in cardiomyocytes without significant immunogenicity. In addition, the combined siRNA polyplexes, that is, C-siRNA with PCM-siRNA and Tat-siRNA, were delivered to the heart at significantly higher levels compared to the unmodified siRNA following systemic administration. These findings indicate that siRNA-pDNA/CD complex may be potentially useful therapeutic tool for cardiomyocyte specific gene therapy.

7.4 Ischemic Myocardium Targeting

Systemic administration of polyplexes targeted to the ischemic myocardium affords significant advantages over local injection. First, targeted polyplexes can be administered immediately following reperfusion via iv injection and do not require a separate procedure with accompanying risks to deliver the polyplex locally. Second, systemic administration allows for easy follow-on dosing to maximize the gene therapy benefit. A primary cardiomyocyte-targeted polymeric gene carrier was developed that enhanced gene transfection in cardiomyocytes using a homing peptide [110]. However, since it may direct the gene to alternative heart tissues as well as the intended ischemic region when administered systemically, future challenges still remain [105]. Recently, a peptide sequence that has high specificity to ischemic myocardium was identified by in vivo phage display in the ischemia/reperfusion (I/R) rat model [112]. In vivo phage display for high-throughput screening, referred to as in vivo biopanning, allows for the screening of a specific peptide that homes organs or tissues in a living animal [113]. This technique is useful for the development of targeted therapeutics, imaging agents, and diagnostic markers in various diseases [114]. Nevertheless, only a few studies have reported targeted delivery of therapeutics by using diseased tissue-targeted peptides, and their main objectives were targeting to tumors or blood vasculature. It has been demonstrated that the ischemic myocardium-targeted peptide (IMTP) could direct specific genes to the ischemic heart and increase the accumulation and transfection of said genes in the ischemic myocardium.

CBA-DAH was chosen as a backbone for the modification with IMTP and d-9-arginine (9R). Since 9R has been known as the most effective peptide for protein transduction as well

as gene transfection, the attachment of 9R to CBA-DAH would increase the transfection efficiency and decrease the amount of polymer required for sufficient transfection [115-117]. Although various polymeric gene carriers have shown evidence of a great potency for cardiovascular medicine, gene therapy for cardiovascular disease still has challenges due to the difficulty in ischemic heart targeting [118]. The targeting effect of IMTP-CD-9R/DNA polyplex and increase in gene expression in the LV in an I/R rat model after a systemic injection has been clearly demonstrated [119]. An ischemic myocardium-targeted gene delivery system was developed by conjugation of IMTP to CBA-DAH polymer, which reduced the required amount of polymer for transfection by the attachement of 9R. Conclusively, IMTP-CD-9R is capable of targeting to the ischemic myocardium and enhances gene expression in the LV in an I/R rat model upon an intravenous injection.

8. Genetically Engineered Cell Delivery

A novel approach to cardiac repair is to combine cellular transplantation and angiogenic gene therapy. The gene carriers that can transduce angiogenic genes into primary myoblasts, however, have not been optimized. While viral vectors, including adenovirus, adeno-associated virus, and retrovirus, have high gene transfection efficiency, they have limited clinical application due to their inherent potential for immunogenicity, tumorigenicity, induction of an inflammatory response, and integration into the host genome [120-122]. Furthermore, long-term over-expression of VEGF via viral vectors has been observed to lead to hemangioma instead of functional vessels in animal models [41, 123]. These findings exclude viruses as clinically viable vectors to transduce angiogenic genes into primary myoblasts. It is therefore necessary to develop a safer and more efficient non-viral gene carrier that can circumvent the limitations of viral vectors.

The in vitro gene expression efficiency and therapeutic effectiveness of polymer mediated transfection of primary myoblasts has been previously assessed [124]. Autologous primary myoblast transplantation may improve the function of infarcted myocardium via myogenesis. In addition, primary myoblasts can carry exogenous angiogenic genes that encode angiogenic factors to promote therapeutic angiogenesis. Two biodegradable poly(disulfide amine)s, CBA-DAH and CBA-DAH-arginine (CBA-DAH-R), were synthesized as polymer carriers for gene delivery. The in vitro time-course and co-culture experiments verified that polymer engineered primary myoblasts have the ability to stimulate endothelial proliferation. These findings confirmed that poly (disulfide amine)s are the safe and feasible polymeric gene carriers to transfect VEGF 165 into primary myoblasts. Polymer engineered primary myoblasts have potential for therapeutic application in the treatment of ischemic heart diseases.

Exogenous primary myoblast transplantation into ischemic myocardium offers many potential benefits for the treatment of myocardial infarction, including improvement and restoration of cardiac function. The improvement in cardiac function is thought to be due to the enhancement of the contractile properties of endogenous cardiomyocytes and the differentiation and proliferation of implanted myoblasts into functional myocardium within ischemic hearts. Recent studies have demonstrated the feasibility of primary myoblast transplantation for the treatment of ischemic heart disease [125, 126]. In addition, it has been

previously reported that VEGF gene therapy, particularly ischemia-inducible VEGF gene therapy, significantly improves myocardial function and reduces left ventricular infarct size following acute myocardial infarction [32, 127]. Excessive VEGF expression, however, has been associated with heart failure and death, due to vascular tumor formation instead of the formation of functioning vessels [128]. Previous work has laid the foundation for current studies demonstrating the functional benefit of this approach for treating acute myocardial infarction in an in vivo rat model [124]. Treatment with poly (CBA-DAH)/VEGF-transfected myoblasts preserved cardiac wall thickness, restored left ventricular function, induced neovascularization and reduced cardiac apoptosis more effectively than untransfected myoblasts. This work demonstrates that combining cellular implantation therapies with gene therapy can potentially increase the efficacy of surgical implantation of cells and produce better patient outcomes.

9. Minicircle DNA encoding VEGF

The application of plasmid DNA-based gene therapy is limited by its inefficient transgene expression. Minicircle DNA was evaluated for efficient VEGF expression in skeletal muscle cells [129]. The VEGF minicircle DNA under control of the SV40 promoter (pMini-SV-VEGF) showed an increased amount of VEGF mRNA and up to 8 times more VEGF expression than a conventional plasmid (pSV-VEGF) in two different skeletal muscle cell lines (C2C12 and L8). Minicircle DNA with different promoters, including the SV40, CMV and chicken β-actin, was tested for VEGF expression in a C2C12 skeletal muscle cell line. The high VEGF expression generated by minicircle DNA stimulated efficient endothelial cell growth in vitro. Furthermore, minicircle DNA showed a higher VEGF expression compared to conventional plasmid in the tibialis anterior muscle of mice. These results suggest that minicircle DNA is an effective gene vector for angiogenic VEGF expression in skeletal muscle and may be useful for treating peripheral arterial disease.

10. Human EPO Gene Delivery

EPO plays a key regulatory role in the formation of new red blood cells (RBCs). EPO may also have a role as a therapeutic agent to counteract ischemic injury in neural, cardiac and endothelial cells [130]. Several reports have demonstrated the capacity of EPO to protect and revascularize the myocardium following ischemic injury [131-133]. One of the limitations preventing the therapeutic application of EPO is its short half-life. The development of a more sustained form of EPO with a longer half-life would remove a significant barrier to its development as a therapeutic agent. Recently, several researchers have focused on the development of an EPO plasmid DNA delivered using either viral or non-viral carriers to promote the prolonged and controlled release of EPO in vivo [134]. Despite widespread use of recombinant human EPO (rHuEPO), several clinical limitations remain, including frequent injections, limited routes of administration, high medical expenditures, development of autoimmune pure red cell aplasia, and impacts on hemoglobin variability [135, 136]. To overcome many of these clinical hurdles, gene therapy providing continuous release has been suggested as an attractive alternative to current intermittently administered erythropoiesis-stimulating agents (ESAs) [137]. The transfection assays of the

pEPO polyplex have been carried out in a variety of cell types and the time-course release of EPO has been analyzed in vitro [138].

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 reendothelialization, vascular-protectant, mobilization of endothelial progenitor cells, and recruitment of stem cells into the zone of damage [132]. Apart from traditional erythropoietic effects, the pleiotropic organ-protective effects of EPO make it a frontline cardioprotective candidate. Higher levels of endogenous EPO have been shown to have protective effects against I/R injury in acute MI in humans [139]. 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 [132, 140]. 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 [141]. To date, little is known about how polymer-mediated phEPO therapy distinctly alters cardiac remodeling in the rat MI model. The sustained release of intramyocardial phEPO gene therapy delivered by ABP polymer may restore heart function and limit pathological cardiac remodeling after MI [142]. Additionally, this finding 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. Intramyocardial EPO gene therapy delivered by the bioreducible ABP polymer demonstrates potential cardioprotective effects on post-infarct cardiac remodeling in rats, compared with treatment of rHuEPO protein and naked phEPO plasmid-alone. The prominent effects of EPO/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.

11. Conclusion

Inter-disciplinary researchers have advanced gene therapy due to its potential advantages over conventional treatment for myocardial ischemic diseases. Angiogenesis-based therapy using angiogenic genes have showed positive effects on the ischemic heart in preclinical and clinical studies. Anti apoptotic genes have also demonstrated the ability to prevent apoptosis-induced cell death by the inhibition of the apoptotic pathway in the ischemic heart. The intracellular delivery of anti-apoptotic genes needs improvement in order to increase the therapeutic efficacy and specificity to the targeted organ. Polymeric gene carries have been developed to substitute viral vectors for their avoidance of immunogenicity and oncogenecity. Although considerable improvements in gene transfer techniques using non-viral vectors have been witnessed during the last decade, the standard requirements for clinical use have not been met in terms of efficiency and specificity. When designing polymeric vectors, studies to overcome subcellular barriers including endosomal escape and nuclear translocation should be considered. Gene delivery is a multi-step process, which

needs the appropriate carriers for each stage. Therefore, plasmid and multi functional polymeric vectors should be rationally designed. The plasmid design should include the introduction of tissue-specific promoters or regulatory promoters. As for the multifunctional polymeric vectors, they should be designed in conjunction with the vector development at the molecular level, taking into account the need to overcome a series of extra- and intra-cellular barriers.

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