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Women and Heart Disease - Physiologic Regulation of Gene Delivery and Expression: Bioreducible Polymers and Ischemia-Inducible Gene Therapies for the Treatment of Ischemic Heart Disease

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Abstract

Ischemic heart disease (IHD) is the leading cause of death in the United States today. This year over 750,000 women will have a new or recurrent myocardial infarction. Currently, the mainstay of therapy for IHD is revascularization. Increasing evidence, however, suggests that revascularization alone is insufficient for the longer-term management of many patients with IHD. To address these issues, innovative therapies that extend beyond revascularization to protection of the myocyte and preservation of ventricular function are required. The emergence of gene therapy and proteomics offers the potential for innovative prophylactic and treatment strategies for IHD. The goal of our research is to develop therapeutic gene constructs for the treatment of myocardial ischemia that are clinically safe and effective. Toward this end, we describe the development of physiologic regulation of gene delivery and expression using bioreducible polymers and ischemia-inducible gene therapies for the potential treatment of ischemic heart disease in women.

Keywords

bioreducible; polymer carriers; ischemia-inducible; gene therapy

2 Introduction

Ischemic heart disease (IHD) is the leading cause of death in the United States today. In 2006, over 250,000 deaths in women were attributable to IHD. This year over 750,000 women will have a new or recurrent myocardial infarction. One of the consequences of non-fatal myocardial infarction is congestive heart failure (CHF), as 46% of women surviving heart attacks will develop CHF within 5 years of the event [1]. Currently, there are over 2.5 million American women living with CHF, and these patients have a 5-year mortality of 50%. CHF accounts for over 4 billion dollars in annual Medicare expenditures [1].

Disparities exist in the diagnosis, treatment, and outcome of cardiovascular disease in women (cRosenfeld 2008). Societal and cultural differences are not to be blamed entirely. Under diagnosis of cardiovascular disease is the responsibility of the physician, the public, and the

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individual, as awareness of the differences in disease symptoms between men and women is crucial in early treatment. These differences in cardiovascular symptoms based on gender are mistakenly dismissed in women as due to stress and fatigue, thereby contributing to a delay in treatment (cMcSweeney 80). Under representation of women in research trials, based on failure to meet enrollment criteria due to age and preserved systolic function, has led to disparities in outcomes for women for congestive heart failure and short and long-term mortality following an ischemic event (11–13 SBCP 2008).

Currently, the mainstay of therapy for IHD is revascularization. Nearly 2,000,000 cardiac catheterizations and 553,000 coronary artery bypass grafting procedures are performed annually [1]. The development of these technologies has led to an improved survival and quality of life for patients with IHD. Increasing evidence, however, suggests that revascularization alone is insufficient for the longer-term management of many patients with IHD. A significant number of women will develop CHF each year following ischemic events to the myocardium despite having received revascularization therapy. In addition, revascularization of ischemic myocardium can result in reperfusion injury, which is associated with a cascade of events promoting additional myocyte loss and detrimental ventricular remodeling [2]. To address these issues, innovative therapies that extend beyond revascularization to protection of the myocyte and preservation of ventricular function are required.

3 Gene Therapy

Developments in genetics and molecular biology have led to a better understanding of the pathophysiology of the progression from ischemic heart disease to CHF. The emergence of gene therapy and proteomics offers the potential for innovative prophylactic and treatment strategies for IHD. Gene therapy is the exogenous introduction of genetic information into cells that results in altered production of proteins to correct or modulate a disease state. The selection of a proper gene transfection method is an important issue in gene therapy.

3.1 Viral Gene Delivery Systems

Vectors for gene delivery must be clinically applicable: safe and effective. Two major types of gene delivery methods are in use today: viral and non-viral gene delivery. Virus mediated gene therapy is based on the use of attenuated or replication-defective viruses and includes the use of retroviruses, adenoviruses, adeno-associated viruses, and herpes viruses. Viral delivery systems are predominantly used, as they are more effective in terms of transfection efficiency, compared to non-viral systems. Viral gene delivery, however, possesses serious risks that limits its clinical use: i) induction of host immune responses against viral components; ii) the risk of potential chromosomal insertion of viral sequences into the host chromosomes, which could lead to undesirable cell transformations, such as the activation of oncogenes and the inactivation of tumor suppressor genes; iii) size limitations of DNA for encapsulation; iv) high cost of production; v) difficulties in quality control [3–7]. It is for these reasons that the development of non-viral systems is becoming increasingly attractive.

3.2 Non-Viral Gene Delivery Systems

Non-viral gene delivery systems offer numerous advantages over viral vectors including: a) excellent safety profiles; b) unlimited DNA size for loading; c) relatively low cost of production; d) a simple quality control process; e) the opportunity for repeated treatment [8]. Plasmid DNA, which encodes information for the expression of one or more therapeutic proteins, is highly negatively charged and has relatively high molecular weight. Delivered by itself, plasmid DNA is subject to nuclease degradation and non-specific binding to positively charged serum proteins [9]. Furthermore, the transport of plasmid DNA is limited due to the charge repulsion between the plasmid DNA and the negatively charged plasma membrane.

These limitations can be overcome with the use of cationic polymer-based carriers that can be utilized to condense plasmid DNA molecules into particles. Requirements for the logical design of cationic polymeric gene carriers include: i) protection of the plasmid DNA from degradation by nucleases, thereby extending the time of biologic activity; ii) promotion of the internalization of complexes on cellular surfaces due to electrostatic interactions; and iii) low toxicity and high gene expression.

3.2.1 Cationic polymers—Polyethylenimine, PEI, is still considered one of the most successful non-viral gene carriers and is used extensively in *in vitro* as well as *in vivo* gene delivery due to its high transfection efficiency and reproducibility [10,11]. PEI possesses high endosomal buffering capacity, which facilitates the endosomal escape of the complexes via the hypothetical proton sponge effect [12]. Clinical use of PEI, however, is still hindered by the high cytotoxicity of the polymer. Although PEIs with lower molecular weight (MW, < 2 kDa) exhibit much lower cytotoxicity, the reduced transfection efficiency of the low MW PEIs limits their use as *in vivo* gene carriers due to poor endosomal release [13–15]. However, the amine groups providing for endosomal release also allow for the conjugation of targeting moieties and shielding agents to overcome the extracellular and intracellular obstacles facing polycationic gene delivery.

3.2.2 Cationic lipids—Several cholesterol based cationic lipids have been generated as gene carriers and have demonstrated high transfection efficiency in mammalian cells [16–18], but most have lacked an effective endosomal escape mechanism such as that found in PEI. Cholesterol uptake is accomplished through receptor-mediated endocytosis and helps to maintain plasma membrane integrity [19]. Most cholesterol is supplied by circulating lipoproteins and while the cholesterol demands of the heart and skeletal muscle are low [20], selective uptake may be mediated by lipoprotein lipase (LPL) [21]. As LPL expression is high within cardiac muscle, it is logical that a cholesterol moiety would enhance transfection within the myocardium over naked DNA while reducing the toxicity seen from other non-viral carriers [22].

3.2.3 Hydrolytic polymers—Modifications to increase polymeric gene delivery efficiency should incorporate molecules that have the ability to discriminate between differences in biologic microenvironments, such that they respond to pH, ionic or redox potentials [23]. These modifications take advantage of changes in pH to assist in cell targeting through the incorporation of acid-labile linkages to facilitate polymer degradation within the local environment of a tumor [24], or to assist in endosomal release of either stabilizing complexes [25–30] or polyplex degradation [14,31,32]. The latter is increasingly being realized as being important as polyplex unpacking is seen as a rate-limiting step for non-viral mediated gene delivery, especially for large polymer constructs [33]. These acid labile polymers may exhibit poor transfection due either to rapid hydrolysis or poor endosomal escape, demonstrating that in the design of hydrolytically degradable carriers, a balance needs to exist between extracellular hydrolytic stability and rapid intracellular degradation. This is further compounded by difficulties in vector handling and post-modifications of these hydrolysis sensitive polymers [32]. A more rational approach is to have the complex escape the endosome and instead decomplex within the cytoplasm.

3.2.4 Bioreducible polymers—Bioreducible polymers use disulfide bonds instead of ester linkages to take advantage of the differences in reductive potential between extracellular and intracellular microenvironments in normal cellular as well as diseased cellular states. The existence of these differences in reductive potential has prompted the design of gene carriers to incorporate disulfide bonds into their structure to facilitate intracellular gene delivery [34–37]. Delivery of nucleotides by means of redox-sensitive gene carriers has included mRNA

(38), antisense oligonucleotides [27,30,39], siRNA and plasmid DNA [40–44]. These studies have incorporated numerous cell lines and several disease states, but until recently have not included cardiac tissues [45,46]. Polyamines with multiple reducible disulfide bonds through amide bond formation have many favorable features. These polymers can form small and stable complexes when combined with plasmid DNA, which facilitates the endocytosis-mediated cellular uptake of the plasmid DNA. They can efficiently protect plasmid DNA from the enzymatic degradation by nucleases until successful delivery into the intracellular cytoplasmic compartment, where the disulfide bonds of the polymer backbone are reduced by the action of reductive glutathione to release the plasmid DNA. The difference in redox potential (100 fold) between the oxidizing extracellular space and the reducing intracellular space makes disulfide bonds unique in cellular biology and in gene delivery systems [47]. The use of reducible polymer systems provides a way to maintain the effective characteristics of polyethylenimine (PEI) based systems and achieve efficient gene expression in vivo, while limiting toxicity. In a preliminary study, disulfide bond–polyamido-ethylenimines (SS-PAEIs) demonstrated significantly lower cellular cytotoxicity than PEI 25K. The reduced toxicity was due to the capacity of the polymer to be cleaved into shorter segments in the reductive intracellular cytoplasm, eliciting a lower toxicity profile compared to PEI. The products from the reductive degradation of the SS-PAEI polymers will be readily eliminated by the in vivo clearance system. The triggered degradation of the SS-PAEIs in the cytoplasm leads to the release of plasmid DNA from the polymer/DNA complexes. This triggered release mechanism allows for improved access of the plasmid to the nucleus, thereby promoting transcription and consequently resulting in an increase in expression of the therapeutic protein.

4. Sex-based cardiac differences

The structure and function of the heart is similar between men and women after puberty [48]. In particular, increases in septal and wall thickness are similar for both men and women as age increases. Left ventricular diameter, however, increases only in men and is attributed to a loss of myocardial mass and myocytes [49]. This loss leads to compensatory left ventricular hypertrophy to maintain ventricular function. These same changes, however, are not evident in aging women. Despite these gender based differences, however, women progressively develop cardiovascular disease as age progresses [50,51].

Differences in hormone levels between the sexes are the most obvious factors for study and have led to theories describing the cardioprotective effects of estrogen and the negative effects of testosterone on cardiac function. These correlations have also been demonstrated in other physiological systems and diseases [52]. Estrogen and androgen receptors are present in male and female myocardium and may act directly or indirectly on transcription, protein kinase activation, and growth factor upregulation [53–55]. Ovariectomized animal models mimic the loss of estrogen seen in post-menopausal women and display an increased risk of cardiovascular disease including heart failure, pulmonary edema, and decreased left ventricular function [56]. While this strongly suggests the role of estrogen in cardioprotection, its mechanism remains elusive.

Evidence points to the ability of estrogens to induce nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in cardiac myocytes. The reduction of levels of estrogen in ovariectomized females may reduce infarct size following myocardial ischemia reperfusion injury [57,58]. Stronger evidence exists for the effect of estrogens on angiogenesis. Ovariectomized females demonstrate a significant reduction in angiogenesis that upon estrogen replacement is restored [59]. Supporting this finding is the experimental observation that the intramuscular injection of estrogen into a rabbit ischemia hind-limb model induces collateral vessel formation [60,61]. Mechanistically, these effects are multi-factorial and may involve up-regulation of growth factors, surface adhesion factors and integrins, as well as inhibition of

apoptosis [63–67]. However, despite the known positive aspects of estrogen on human cardiac structure and physiology, there remain doubts as to the clinical benefit of estrogen replacement therapy for women with regard to the prevention and outcomes of cardiovascular disease.

5 Neovascularization

Neovascularization is the process of new blood vessel development from preexisting vasculature [68]. Development of collateral vessels is a critical biological response to tissue hypoxia caused by coronary artery disease [68]. The growth of collateral coronary vessels occurs naturally in the setting of native coronary artery stenosis and can dramatically change the natural history of coronary disease. Adequate collateralization can overcome severe stenoses, relieving ischemia, provided that the narrowing of the native artery does not progress too quickly. In the case of acute myocardial infarction, however, thrombus formation occurs quickly, while the collateralization process requires weeks to months. Over the past 15 years, our knowledge of the molecular processes that affect angiogenesis and collateralization has increased significantly.

5.1 Vascular Endothelial Growth Factor

Studies elucidating the molecular mechanism of vascular development initially focused on discovering the mitogens for the key cell types: endothelial and vascular smooth muscle cells. These studies identified vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factors (PDGF), angiopoietins and their cognate receptors. A number of growth factors, including the vascular endothelial growth factor (VEGF) family, have been identified as contributing to the process of angiogenesis. VEGF is a potent mitogen for endothelial cells in vitro. In vivo, VEGF stimulates capillary formation and increases vascular permeability. The ability of VEGF to stimulate angiogenesis has been demonstrated by several groups in a variety of animal models [69–71]. While other agents may be applicable in this setting, they suffer from significant limitations compared to VEGF. Fibroblast growth factor (FGF), for example, is a potent stimulator of endothelial cell proliferation but FGF also stimulates vascular smooth muscle cells (VSMC) and fibroblast proliferation. This increases the possibility that FGF can accelerate VSMC proliferation in atherosclerotic plaques. Also, in contrast to VEGF, FGF has no effect on vascular permeability [72]. Subsequent gene targeting experiments showed that these signaling pathways are essential for mammalian vascular development, and supported a simplified paradigm of blood vessel formation. VEGF and FGF stimulate endothelial proliferation and sprouting. PDGF secreted by endothelial cells induces recruitment of the vascular media. Expression of angiopoietins and matrix proteins triggers maturation and remodeling of the blood vessel.

5.2 Unregulated Expression of VEGF

Over-expression of VEGF can lead to several important problems, including the formation of vascular tumors, or angiomas [73]. This is of particular concern within the heart, as angiomas constitute space-occupying lesions that lead to heart failure and death [74]. Another problem is that unregulated normoxic VEGF activity has been shown to promote progression of atherosclerotic lesions, a significant concern in treating patients with IHD [75]. Finally, systemic expression of VEGF could potentially exacerbate diabetic retinopathy or promote angiogenesis of peripheral tumors [76]. These problems suggest that in order for gene therapy to become acceptably safe and widely applicable, regulation of the expression of the gene product will be essential. At the University of Utah, we have developed a means to regulate expression of our gene therapy products by the in situ oxygen tension within the tissue. Once the gene therapy directed neovascularization has restored normal tissue oxygen tension, no significant additional protein is expressed. Peripheral gene expression is negligible, as normal tissue oxygen tension at distant sites inhibits expression of the neovascularization peptide. This

temporal and regional control of gene expression expands the safety profile for gene therapy mediated neovascularization within the myocardium.

5.3 Neural Guidance Cues and Vascular Development

Research into the biology of neovascularization has now evolved towards understanding the vascular system as an integrated network. Blood vessels are catalogued by their luminal caliber, the organs that they supply, and by whether they are arteries or veins. The differences amongst blood vessels are utilized to form an organized pattern that is reproduced within and between vertebrate species. Molecular signals such as ephrin and the Eph genes distinguish arterial from venous endothelial cells and, when disrupted, result in an immature vascular pattern. Increasingly, the concept of vascular development has shifted towards a paradigm of integrating circuitry, similar to the development of the nervous system. In both neural and vascular networks, a common problem must be tackled. Central axial structures send sprouts along predetermined and often shared trajectories to their distal destinations. In recent years, it has become clear that many of the molecular programs that guide the patterning of the neural network, also play a role in promoting neovascularization.

6. Research and Development Goals

The goal of our research is to develop therapeutic gene constructs for the treatment of myocardial ischemia that is clinically safe and effective. To meet this goal, we have sought to: 1) develop non-viral gene carriers, i.e. lipopolymers, which can promote transfection of the myocardium; 2) make expression of the gene product subject to the same ischemia control mechanisms which are present in vivo; 3) develop novel genes for the treatment of myocardial ischemia.

7 Development of Novel Lipopolymers

Our initial efforts were directed towards developing a novel lipopolymer carrier for use in cardiovascular gene delivery. Towards this end, we developed a new gene carrier system, TerplexDNA [77]. TerplexDNA is composed of plasmid DNA (pDNA), stearyl-poly-L-lysine (stearyl-PLL), and low density lipoprotein (LDL). LDL binds to vascular endothelial cells and smooth muscle cells and aids in facilitation of receptor-mediated endocytosis, allowing for high transfection efficiency to smooth muscle cells in vitro. We found that TerplexDNA mediated delivery of plasmid VEGF administered at the time of coronary occlusion improves left ventricular function and reduces left ventricular dilation [78].

7.1 Water-Soluble Lipopolymer

Following these studies, we based our second generation polymer on a branched polyethylenimine (bPEI) backbone, as bPEI can effectively escape from the endosomal compartment due to the hypothesized proton-sponge effect. To reduce the cytotoxicity of high molecular weight bPEI and enhance the transfection efficiency of low molecular weight bPEI, we developed a water-soluble lipopolymer (WSLP), in which cholesterol was conjugated to bPEI (1800 Da) [13]. The cholesterol addition provided extra condensation capabilities through the formation of stable micellular complexes in aqueous solutions and enhanced permeability through cell membranes due to the high lipoprotein lipase expression within cardiac tissue. The transfection efficiency of myocardium by WSLP is superior to PEI1800, PEI25k and Naked DNA. The reason is that WSLP has the following advantages: 1) a higher transfection efficiency and longer gene expression in myocardium than naked DNA and bPEI; 2) low cytotoxicity; 3) it does not integrate the plasmid into the host chromosome, which minimizes possible oncogene activation or tumor suppressor gene inactivation; 4) it does not induce a

cellular immune response. These findings indicate that WSLP can be applied to in vivo gene therapy for the treatment of ischemic heart disease.

7.2 Development of Ischemia-Inducible Gene Therapy

Another important consideration for gene therapy is the construction of an effective therapeutic gene. Our initial work demonstrated the therapeutic potential of the gene encoding vascular endothelial growth factor (VEGF) as a treatment for myocardial ischemia. This initial work was done with an unregulated, constitutive promoter. Unregulated VEGF mediated angiogenesis, however, has the potential to promote tumor growth [79], accelerate diabetic proliferative retinopathy [80], and promote rupture of atherosclerotic plaque [81]. To be safe and effective, gene therapy with VEGF must be regulated. Natural expression of VEGF is controlled by its own hypoxia-driven regulatory system at two different levels: transcription and translation. The VEGF promoter has a hypoxia-responsive element that binds to HIF-1 and activates the transcription of the gene under conditions of ischemia. 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.

Several ischemia-inducible genes including erythropoietin (Epo) have hypoxia-responsive elements in their promoters and enhancers. By employing the Epo enhancer for the regulation of the VEGF gene, we are able to mimic the physiologic, in vivo regulation of VEGF expression [82]. To test this hypothesis, we developed an ischemia-inducible VEGF gene therapy system using the Epo enhancer and inserted into our water-soluble lipopolymer (WSLP) gene carrier. pEpo-SV-VEGF or pSV-VEGF_{Epo} was constructed by insertion of the Epo enhancer upstream of the Simian Virus 40 (SV40) promoter or downstream of the poly(A) signal of pSV-VEGF. After transfection with the plasmid/WSLP complexes, cells were incubated under normoxic or hypoxic conditions for 24 or 48 h. The medium was harvested and enzyme-linked immunosorbent assay (ELISA) was performed to measure the level of the secreted VEGF protein. After 24 h of incubation, pEpo-SV-VEGF expressed approximately three fold higher levels of VEGF proteins under hypoxic conditions than under normoxic conditions. After 48 h of incubation, pEpo-SV-VEGF expressed approximately 10 fold higher levels of VEGF proteins under hypoxic conditions than under normoxic conditions. Without the Epo enhancer, pSV-VEGF did not enhance the production of VEGF under hypoxic conditions. Therefore, the induction of VEGF expression was specific for hypoxic cells. In addition, the VEGF protein, which was produced from the Epo-SV-VEGF-transfected and hypoxia-incubated cells, was able to enhance the proliferation of endothelial cells. Based on these findings, we studied in vivo VEGF gene expression. The results showed that the expression level of VEGF by pEpo-SV-VEGF in ischemic myocardium was three times higher than in normal myocardium. Further, the pSV-VEGF construct could not significantly induce VEGF expression in normal or ischemic myocardium. Therefore, these results indicate that a hypoxia regulated gene expression system is applicable to ischemic tissue in vivo to minimize nonspecific gene expression.

7.3 Post-Transcriptional Regulation of Gene Expression

These ischemia-inducible promoter systems, as represented by the pEpo enhancer, regulate gene expression at the transcriptional level. Gene expression, however, can also be regulated at the post-transcriptional, i.e. the translational, level. The regulation at the post-transcriptional, or translational, level can be achieved mainly by controlling the mRNA stability. The Epo 3'-untranslated region (UTR) is known to stabilize the Epo mRNA under hypoxic conditions [83]. The erythropoietin mRNA binding protein (ERBP) binds to the Epo 3'-UTR, thereby stabilizing and increasing the half-life of the mRNA. This leads to a four fold higher steady state level of the Epo mRNA and subsequent higher levels of translation of protein. To regulate

the gene expression in response to hypoxia, therefore, the Epo 3'-untranslated region (UTR) was cloned by RT-PCR. The WSLP/plasmid complexes were transfected to 293 or A7R5 cells and the cells were incubated under normoxic or hypoxic conditions. The results showed that both the Epo enhancer and the Epo 3'-UTR increased the target gene expression under hypoxic conditions [84]. The pEpo-SVLuc- EpoUTR construct showed the highest luciferase expression. When the plasmids were reconstructed with the gene for VEGF replacing luciferase, the VEGF expression by pEpo- SV-VEGF-EpoUTR showed the highest levels of VEGF gene expression in the hypoxic cells. The results showed that the UTR increased the VEGF gene expression specifically under hypoxic conditions and that the combination of the Epo enhancer and 3-UTR was the most effective construct for gene expression under conditions of hypoxia. With further optimization of the system, the combination of the Epo enhancer and the Epo 3'-UTR will be useful for the development of gene therapy for myocardial ischemia.

7.4 RTP801 Promoter

The RTP801 promoter became the emphasis of research after it was demonstrated to regulate transcriptional activation under conditions of cellular hypoxia [85]. We set out to characterize the RTP801 promoter and identified a cis-regulatory element responsible for its hypoxic induction [86]. Deletion analysis of the RTP801 promoter confirmed a cis-regulatory element between -495 and -446 and a negative regulatory element between -545 and -496. Sequence analysis of the purported cis-regulatory element showed a potential Sp1 binding site and site-directed mutagenesis was performed to further evaluate its effect under hypoxia. Mutation of the site abrogated any control under hypoxia and Sp1 was hypothesized to mediate hypoxia induction in conjunction with HIF-1 α but the actual mechanism of action remains unidentified. The use of the RTP801 promoter for ischemia inducible therapy may benefit premenopausal women as estrogen increases Sp1 activity. As it was proven to be a stronger promoter than pEpo-SV-VEGF, we set out to provide evidence for its usefulness in ischemia-inducible gene therapy systems. Following the development of our novel lipopolymer carriers and ischemia-inducible promoters, we studied the performance of these constructs in vivo. An acute myocardial infarct model was used to compare the morphological and functional changes within the myocardium following treatment with our water soluble lipopolymer (WSLP) containing either the ischemia-inducible (RTP801) or constitutively expressed (SV) gene for VEGF. RTP801-VEGF gene therapy significantly reduced myocardial infarct size compared to constitutively expressed gene therapy [87]. The use of the RTP801 promoter for ischemia inducible therapy may benefit premenopausal women as estrogen increases Sp1 activity [88].

7.5 Development of Bio reducible Polymer Carrier for Ischemia-Inducible Gene Therapy

Our next generation polymer, reducible poly(amido ethylenimine) (SS-PAEI), is a biodegradable polymer [90]. These polymers are promising due to their (1) facile synthesis, (2) stability in an aqueous environment for purification, (3) endosomal buffering capacity, (4) small particle formation when complexed with plasmid DNA (pDNA), (5) relative low toxicity and high mediated gene expression compared to PEI, (6) low serum interactions in vitro and (7) the ability to take advantage of the high physiological redox potential which exists within the cell. Controlled degradation of these polymers allows for more efficient unpacking and release of genetic material into the cell, thereby improving efficiency of nucleic acid delivery and reducing carrier induced toxicity. Reducible poly(amido ethylenimine) (SS-PAEI) synthesis produces polymers with a low polydispersity and stable complex formation with pDNA until introduced into a reductive environment, such as the one which exists inside living cells. Evaluations were performed on the myocardial cell line, H9c2, using a luciferase reporter plasmid, a toxicity assay, and a plasmid driving green fluorescent protein (GFP) expression in order to show transfection efficacy. Luciferase reporter gene expression of SS-PAEI on H9C2 cells demonstrated low toxicity and high transfection efficiency when compared to bPEI25k [45]. In order to accurately assess the transfection efficiency of the SS-PAED/pDNA system,

CMV-GFP was used for fluorescence-activated cell sorting (FACS) analysis. SS-PAEI positively transfects up to $57 \pm 2\%$ of H9C2s at 12:1 w/w compared to bPEI at $11 \pm 1\%$. To our knowledge, this is the highest level of GFP expression mediated by a non-viral carrier system reported in cardiac cells to date. The fact that such expression is obtained in a cardiac cell line reveals the potential of this polymer for in vivo cardiovascular therapy. Previous work in our laboratory using water soluble lipopolymer (WSP) at 200ug/ml produced significantly higher gene expression than naked pDNA and bPEI/pDNA in the same model [83]. We initially decided to perform dose response injections with our SS-PAEI/RTP-VEGF system starting at 100 μ g RTP-VEGF and then increasing the dose until VEGF expression was apparent [45]. SSPAEI/RTP801-VEGF at 12:1 w/w showed the highest overall VEGF expression, nearly 4 fold higher than the RTP801-Luc control and 2 fold higher than the WSP positive control at half the dose of the VEGF gene. We believe this is the first report to use reducible polymers for therapeutic gene delivery of VEGF in vivo. SS-PAEI is an efficient non-viral gene delivery vehicle that not only is nontoxic but also mediates high levels of gene expression in vitro and in vivo. This significantly higher transfection efficiency should significantly reduce any of the remaining objections to the use of polymers as delivery vehicles for gene therapy.

7.6 Novel genes for treatment of myocardial ischemia – Netrins

The field of vascular development has now evolved towards understanding the system as an integrated network. Blood vessels are catalogued by their luminal caliber, the organs that they supply, and by whether they are arteries or veins. The differences amongst blood vessels are utilized to form an organized pattern that is reproduced within and between vertebrate species. Molecular signals such as ephrin and Eph genes distinguish arterial from venous endothelial cells and when disrupted result in an immature vascular pattern. Increasingly, the view of vascular development has shifted towards concepts of integrating circuitry, reminiscent of how neurobiologists view the neural system. In both neural and vascular networks, a common problem must be tackled. Central axial structures send sprouts along predetermined and often shared trajectories to their distal destinations. In recent years, it has become clear that many of the molecular programs that guide the patterning of the neural network, also play a role in angiogenesis. Netrin-1 and Netrin-2 were isolated because they stimulated neural outgrowth. We hypothesized that if netrins had an analogous function in the vascular system, then netrins would stimulate angiogenesis. We showed that Netrin-1 is a potent mitogen and chemotactic agent for human microvascular endothelial cells (HMVECs) with a specific activity comparable to VEGF, and has little influence on endothelial adhesion [90]. In addition, we did not detect the expression of the attractive netrin receptors DCC and Neogenin by Northern and Western blot analysis in HMVECs suggesting that another receptor is responsible for the mitogenic and chemotactic activities of Netrin-1 on endothelial cells. Since netrins stimulate axonal sprouting, we measured vascular sprouting using in vivo models of angiogenesis. In chorioallantoic membrane (CAM) and the corneal micropocket assays, we showed that Netrin-1 was angiogenic, inducing vascular sprouting. The effects of Netrin-1 and VEGF were additive if not synergistic.

Netrins promote therapeutic angiogenesis in models of peripheral vascular disease. Before attempting to investigate the role of Netrins in promoting therapeutic angiogenesis in the heart, we sought to demonstrate proof of principle in far less complex model. We therefore compared netrins with VEGF in their ability to promote angiogenesis and re-perfusion in a murine model of hindlimb ischemia [91]. The iliac artery of FVB/NJ mice was ligated, resulting in severe vascular perfusion defects. Blood flow was measured with laser Doppler imaging and quantitated as the ratio of ischemic to nonischemic limbs. Expression of Netrin-1, Netrin-4 and VEGF cDNAs were driven by a CMV enhancer and RSV promoter characterized previously in preclinical and clinical trials of VEGF [92–96]. The base expression vector (empty vector) was used as a control. All constructs were delivered locally into the ischemic gastrocnemius

muscle at 0, 7, 14 and 21 days following induction of ischemia. Imaging revealed that hindlimb perfusion was significantly improved 7 days post-surgery in mice injected with Netrin-1, Netrin-4 or VEGF constructs. Serial Doppler measurements demonstrated continued improvement in limb perfusion with the greatest effect at 28 days after induction of ischemia. Histological analysis after day 28 showed a significantly greater (>2-fold) capillary density and reduced fibrosis in the Netrin-1-, Netrin-4- and VEGF-treated animals.

8 Conclusion

The increased incidence of cardiovascular disease and the poorer prognosis following ischemic events in women indicate that there is a vital need for focused attention on the unique aspects of cardiovascular disease which affect women. While significant progress has been made in improving the treatment of cardiovascular disease for both sexes, research into the use of gene therapy for the treatment of cardiovascular disease needs to account for possible differences in outcome based on gender. Clear benefits have been seen using novel non-viral gene therapies for restoring cardiac function, but additional research needs to be undertaken to better understand how the efficacy of gene therapy is affected by these sex-based differences.

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