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Kiyotake Ishikawa *Editor*

Cardiac Gene Therapy

Methods and Protocols

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Preface

Rapid evolutions in vector technologies and identification of key molecular targets have facilitated the use of gene therapy as a vital approach for treating cardiovascular diseases. In the past decade, there has been substantial progress in clinical translation of cardiac gene therapy. Nevertheless, recent early clinical trials using gene therapy as a therapeutic approach to improve heart failure have shown neutral results, and the difficulty of transferring the genes to human hearts has become ever more recognized. Efficient, cardiac-specific, and safe vectors, as well as refined vector delivery methods, are key for successful cardiac gene transfer and eventually for improving patients' outcomes. Newer vectors and more efficient vector delivery methods have the potential to dramatically improve gene transduction efficacy, while novel gene manipulation techniques enforce the therapeutic power and broaden disease targets.

The aim of this book is to provide methodological information on cardiac gene delivery from classic to state-of-the-art technologies and techniques. Detailed and practical protocols described in this volume will be valuable tools for molecular biologists and physiologists in the cardiology field to conduct cardiac gene transfer research, which will ultimately lead to further advancements in the field.

I thank all expert authors for their dedication in describing step-by-step methodologies that will undoubtedly lead to successful cardiac gene therapy. I am very grateful to Dr. Roger J. Hajjar (Icahn School of Medicine at Mount Sinai) for assisting me with the organization of the contents and also for contributing a number of chapters himself. Lastly, I would like to thank John M. Walker, the series editor, who provided me with this opportunity and guiding the volume's preparation process. We hope that the readers find *Cardiac Gene Therapy: Methods and Protocols* to be a useful reference for conducting and improving their projects.

New York, NY, USA *Kiyotake Ishikawa*

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Part I

Overview

Chapter 1

Current Methods in Cardiac Gene Therapy: Overview

Kiyotake Ishikawa and Roger J. Hajjar

Abstract

During the last decade, there has been a significant progress toward clinical translation in the field of cardiac gene therapy based on extensive preclinical data. However, despite encouraging positive results in early phase clinical trials, more recent larger trials reported only neutral results. Nevertheless, the field has gained important knowledge from these trials and is leading to the development of more cardiotropic vectors and improved delivery systems. It has become more evident that humans are more resistant to therapeutic transgene expression compared to experimental animals and thus refinement in gene delivery tools and methods are essential for future success. We provide an overview of the current status of cardiac gene therapy focusing on gene delivery tools and methods. Newer technologies, devices, and approaches will undoubtedly lead to more promising clinical results in the near future.

Key words Cardiac gene therapy, Heart failure, Adeno-associated vectors, Gene delivery, Surgical delivery, Percutaneous delivery, Cardiotropic, Promoters

1 Introduction

From the time I wrote a chapter in this book series in 2003 describing the cardiac gene transfer methods in rodents [1], there has been tremendous progress in the cardiac gene therapy field towards clinical translation. After an early disappointment from the neutral phase II–III angiogenic gene therapy trials using plasmid DNA and adenoviral vectors [2], the field has quickly shifted to more efficient vectors and delivery methods to improve gene transfer efficacy. Application of recombinant adeno-associated virus (rAAV) for cardiac gene delivery is a representative technological advance that enables long-term, efficient, and homogeneous cardiac gene transduction. Numbers of preclinical studies have demonstrated efficient transgene expression and therapeutic efficacy using this vector [3, 4] which led to the initiation of early phase clinical trials [5]. However, after much promise in these early phase trials [5–7], a more recent larger trial reported only neutral results [8]. Similarly, another phase II clinical gene therapy trial utilizing transcatheter

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endocardial injection of plasmid DNA failed to meet the primary efficacy endpoint [9]. These results will delay the application of cardiac gene therapy in daily clinical practice; however, we have gained important knowledge to move forward. Cardiac samples obtained from patients who underwent cardiac transplantation after the rAAV gene therapy has informed us that the vectors indeed transduce the human heart [6]. Notwithstanding, the viral uptake within the myocardium was much lower in humans compared to animal studies which only corresponds to less than 1 % of cardiomyocytes being infected [10]. These results direct us to refine our methods of cardiac gene transfer including a search for better vectors, more robust delivery systems and novel targets. This book has a timely focus on these methodologies to further improve cardiac gene transduction, and covers various novel techniques to produce better vectors that specifically and efficiently target the heart. In this chapter, we provide an overview of currently available cardiac gene delivery vectors and delivery methods.

2 Vectors

One of the most important factors for successful gene therapy is the choice of vectors. Vectors determine the efficiency of transduction, tropism to the targeted tissues, degree of inflammation, and length of transgene expression. Despite the progress in the identification of promising targets for the treatment of a number of cardiovascular diseases, the targeted delivery of therapeutic nucleic acids yet remains a formidable hurdle especially in advanced mammalians. Nonetheless, over the past years, considerable advances have been made in developing and improving several vector platforms. Broadly, these vectors can be classified into two groups: nonviral vectors and recombinant viral vectors. Each of these vector systems has its own set of advantages and disadvantages, and we will briefly discuss the main vectors currently employed in cardiovascular gene therapy.

2.1 Nonviral Gene Delivery

Naked plasmid DNA has been the predominant vector used in the previous cardiac gene transfer trials that have employed nonviral vectors, with only a few trials using lipofection [11]. The major advantages of plasmid DNA include (1) the ease of large scale production, (2) the near absence of a DNA size limit, and (3) the limited innate, cellular and humoral immune response. The lack of a significant humoral immune response against the vector is a great advantage that allows repeat vector administration, which is one of the major limitations of viral vectors. However, repeat vector administration comes with an appreciable risk of serious adverse events due to the administration procedure that often requires invasive procedures. Unfortunately, the Achilles heel of plasmid DNA

as a gene delivery vehicle remains the low transfection efficiencies [12]. Innate immune response to plasmid DNA is considered moderate and can also reduce transfection efficiency [13]. These limitations clearly indicate the necessity of a major breakthrough to improve transfection efficiency to fully realize the potential of plasmid DNA gene transfer.

Recently, promising new nonviral gene transfer methods have emerged. These approaches include modified mRNA [14] (modRNAs) and exosome [15] mediated gene delivery. The use of modified mRNAs has two main advantages: (1) ModRNAs, unlike unmodified nucleic acids, do not bind to Toll-like receptors [16], which could trigger apoptosis of the transfected cells. As a result, modRNAs can transfect the cells very efficiently. (2) Because mRNAs are translated in the cytoplasm, they do not need to be imported into the nucleus for transgene expression, which poses a formidable hurdle for transfection with DNA. ModRNAs trigger high-level transgene expression, and unsurprisingly, transgene expression is relatively short-lived, 2–6 days [14, 17]. Depending on the application, this short, pulse-like expression can be either disadvantageous or beneficial. For example, whereas the short-term expression of proteins deficient in inherited cardiomyopathies would most likely have no long-term therapeutic benefit, the short-term expression of, for instance, growth factors and stem cell recruiting factors [18] might not only be therapeutically optimal but also safer. Recently, Turnbull et al. have shown that modRNA mixed with nanoparticles delivered by direct injection into the myocardium or by intracoronary fashion can induce expression as fast as 20 min following delivery in rodent and in pig hearts [19]. Thus, for short-term and rapid expression, modRNA offers a safe and reliable delivery system to the myocardium.

2.2 Viral Vectors

Recombinant viral vectors are often very efficient in delivering therapeutic genetic material to the targeted cells compared to nonviral vectors. They all have their own characteristics and appropriate vector selection is one of the key components for successful cardiac gene transfer.

To date, the majority of virus-mediated cardiovascular clinical gene therapy trials have used adenoviral vectors. This vector has the advantage of transducing a broad array of cell types, including cardiomyocytes, with a high transgene expression, although transient. However, adenoviral vectors do not have cardiac tropism and the transgene expression cannot be restricted to certain tissues or cell types unless targeted specifically. The most significant limitation of adenoviruses for cardiovascular gene therapy is however, that they trigger a strong immune response [20, 21]. The so-called first-generation adenoviral vectors, which are deficient in only one viral gene (usually E1), trigger a strong cellular immune response [21], presumably as a result of the expression of adenoviral

proteins. However, even after removing most of the viral gene, i.e., gutless adenoviral vectors, a strong innate immune response against the adenoviral capsid was triggered [22], a risk not to be taken lightly in cardiac gene therapy.

Lentiviral vectors have been used experimentally in preclinical cardiac gene therapy studies [23]. In contrast to γ-retroviral vectors, lentiviral vectors can transduce nondividing cells such as cardiomyocytes. Moreover, long-term expression can be achieved in both nondividing and dividing cells, because they integrate their genetic material into the host genome. The immune response is in general moderate [20], but similar to adenoviral vectors, lentiviral vectors have no specific tropism to cells of the cardiovascular system, which likely will require intramyocardial injection as a vector delivery method when targeting cardiac cells. Moreover, lentiviral vectors can cause insertional mutagenesis through the random integration of DNA into the host genome, raising concerns for the aberrant expression of important genes and to tumorigenesis. These limitations have restricted lentivirus use as a vector for in vivo cardiac gene transfer, and it has been mainly used in ex vivo gene transfer to reprogram cells or to induce cardiac progeny in stem cells [23].

AAVs are one of the most promising gene delivery platforms for cardiac gene therapy. AAVs are small, non-enveloped, single-stranded DNA viruses that are nonpathogenic in general. Both dividing and nondividing cells can be transduced by rAAVs and they can trigger long-term transgene expression even in the absence of genome integration in postmitotic tissues, such as the myocardium. One of the main advantages of rAAV vectors for cardiac gene therapy is that multiple AAV serotypes display natural tropism for cardiomyocytes [24, 25]. In small animal models of cardiac diseases, this allows the systemic administration of rAAVs to efficiently transduce the myocardium. Unfortunately, the cardiac tropism of present AAV serotypes and variants is not perfect. As a result, in large animal models—and most importantly in humans—rAAVs carrying therapeutic genes need to be delivered regionally. The cellular immune response against rAAVs is not very strong. In clinical trials using the hepatotropic AAV serotype 8 to deliver factor IX to treat hemophilia B, two patients experienced a transient transaminase increase, putatively as a result of an anti-AAV immune response [26], but the liver transaminase levels rapidly returned to normal after a short regimen of immune suppression. Interestingly, a cellular immune response has not been detected in more than 300 patients in the CUPID 1 and 2 trials [7]. However, despite the limited cellular immune response against rAAVs, the presence of antecedent neutralizing antibodies emerged as a significant obstacle to the broad application of AAV gene therapy. Preexisting neutralizing antibodies against the naturally occurring serotypes, presumably a result of a prior infection with wild-type AAVs, can significantly reduce the transgene efficacy. In fact, more than half the patients (up to 80% in certain regions) who could have been