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Original article

Targeted high-efficiency, homogeneous myocardial gene transfer

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Abstract

Myocardial gene therapy continues to show promise as a tool for investigation and treatment of cardiac disease. Progress toward clinical approval has been slowed by limited *in vivo* delivery methods. We investigated the problem in a porcine model, with an objective of developing a method for high efficiency, homogeneous myocardial gene transfer that could be used in large mammals, and ultimately in humans. Eighty-one piglets underwent coronary catheterization for delivery of viral vectors into the left anterior descending artery and/or the great cardiac vein. The animals were followed for 5 or 28 days, and then transgene efficiency was quantified from histological samples. The baseline protocol included treatment with VEGF, nitroglycerin, and adenosine followed by adenovirus infusion into the LAD. Gene transfer efficiency varied with choice of viral vector, with use of VEGF, adenosine, or nitroglycerin, and with calcium concentration. The best results were obtained by manipulation of physical parameters. Simultaneous infusion of adenovirus through both left anterior descending artery and great cardiac vein resulted in gene transfer to 78±6% of myocytes in a larger target area. This method was well tolerated by the animals. We demonstrate targeted, homogeneous, high efficiency gene transfer using a method that should be transferable for eventual human usage.

Keywords: Gene therapy; Myocytes; Capillary permeability; Regional blood flow

1. Introduction

Gene therapeutics have tremendous potential to revolutionize treatment of cardiac diseases, but clinical successes have come more slowly than originally predicted. Problems limiting gene transfer efficacy include inadequate delivery to the target tissue, loss of therapeutic effect, and negative interactions with the host immune system. Of these, the delivery issue is fundamental, since all else is irrelevant if the gene never reaches the target. Previously reported myocardial delivery methods include intramyocardial injection [1], coronary catheterization [2,3], pericardial delivery [4,5], ventricular cavity infusion during aortic cross-clamping [6,7], and perfusion during cardiopulmonary bypass [8,9]. Each of these methods is limited by either efficacy or tolerability. The best method reported so far is a cross-clamp protocol where hamsters were cooled to 18 °C during a 5 min infusion of histamine and adenovirus, resulting in gene transfer to 77% of cardiac myocytes [10]. These rigorous conditions illustrate the difficulty in obtaining reasonable levels of myocardial gene transfer. The success of the hamster model provides an opportunity to investigate gene transfer effects in rodents and small mammals, but the ability to translate this delivery method for use in large mammals or humans has never been demonstrated.

We previously reported the role of several parameters relevant to gene transfer efficiency in rabbit *ex vivo* and *in vitro* models [11–13]. The goal of the current report was to build an effective myocardial gene transfer method by sequentially exploring these variables in the large mammal *in vivo* environment. We also monitored general parameters of animal tolerance with the overall goal of defining conditions that could eventually be used in humans.

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2. Methods

2.1. Viral vectors

Adβgal was a serotype 5 adenovirus deleted of the E1 and E3 genes, containing the *Escherichia coli lac Z* gene driven by the cytomegalovirus immediate early promoter. Adenoviruses were expanded in HEK-293 cells and purified by passage through Adenopur columns (Puresyn, King of Prussia, PA). The concentrated virus supplemented with 10% glycerol was dialyzed overnight against PBS with 1 mM MgCl2, and stored at −80 °C until use. Virus particle concentration is calculated from the absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}). The infectious titer is determined by plaque assay. Transgene expression is confirmed by infection of a Cos-7 cells, a non-permissive cell line. The absence of replication competent virus is confirmed by pseudo-plaque assay on Cos-7 cells, documenting the absence of cytolytic activity.

Adeno-associated virus (AAV)2-βgal and AAV6-βgal were serotype 2 and pseudotype 6, respectively. Both contained the *E. coli lac Z* gene driven by the cytomegalovirus immediate early promoter. AAV2-βgal was made by triple transfection as previously reported [14]. AAV6-βgal was amplified using the same process, except the AAV-6 capsid gene was used rather than the AAV-2 capsid gene [15]. Both AAV were purified by sequential passage through an iodixanol step gradient and a sepharose Q ion exchange column. AAV stocks were stored at −80 °C until use. The adeno-associated viruses were donated by Genzyme Corp. (Boston, MA).

2.2. Gene transfer procedure

Eighty-one 1–2 month old pigs weighing 5–7 kg were included in this study. The pigs received sildenafil 25 mg orally, 30 min before cardiac catheterization. General anesthesia was induced with ketamine/xylazine/telazol, and the animals were intubated. Anesthesia was maintained with isoflurane. After surgical access to the neck vessels, guiding catheters were introduced through right carotid artery and/or jugular vein into the left anterior descending coronary artery (LAD) and/or the coronary sinus, respectively. Through the guiding catheters, 2.7 Fr balloon catheters were inserted into the middle portion of LAD just distal to the second diagonal branch and/or the great cardiac vein (GCV) as noted in the individual protocols. The LAD and/or GCV balloons were expanded to 3 Atm for the duration of pretreatment and virus solution infusions as indicated in the individual delivery protocols. The baseline protocol delivered to the LAD alone, so coronary sinus and GCV catheters were not used. Baseline pretreatment consisted of vascular endothelial growth factor (VEGF) 0.5 μg/ml, nitroglycerin (TNG) 250 μg/ml, adenosine 5 mg/ml and 1.0 mM calcium concentration in 10 ml of Krebs's solution infused over 3 min. After pretreatment, 6×10^{10} pfu (3×10^{12} vp) Adβgal or 3×10^{12} vp AAVβgal were delivered in 12 ml of Krebs' solution containing the same concentrations of adenosine, calcium and TNG as the pretreatment. VEGF was included in the pretreatment but not in the virus delivery solution. All solutions were passed through 0.2 μm filters prior to use. Virus delivery occurred over 2 min at a coronary flow rate of 6 ml/min. During delivery, heart rate, blood pressure, end-tidal carbon dioxide and pulse oximetry were monitored. The baseline protocol was performed in 6 animals. All other protocols were tested in 3 animals with exception of the combined LAD and GCV delivery protocol, which was tested in 6 animals.

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocol was approved by the Institutional Animal Care and Use Committee.

2.3. Evaluation of transgene efficiency

Five days after the procedure, animals were sacrificed. The hearts were removed and rinsed with ice-cold phosphate buffered saline (PBS). For histological analysis, the heart was cut into three sections: right atrial and ventricular free wall, left atrial and ventricular free wall, and atrial and ventricular septa, and then the sections were sliced at approximate intervals of 3–5 mm. The tissues were fixed and stained with X-gal using conventional methods [11,16]. X-gal staining was always performed at pH 8.0 to minimize non-specific staining [17].

Two measures were used to define the level of in vivo myocardial gene transfer. Measurement of the gross dimensions of the X-gal stained blue volume provided an idea of the overall quantity of tissue involved, and calculation of the percentage of blue cells on microscopic analysis gave an idea of the gene transfer efficiency within the grossly blue tissue. Digital photographs of the gross tissues were taken to quantify the gene transfer tissue volume. The volume of blue tissue was obtained by measurement of blue surface area and depth of penetration after X-gal staining. Digital pictures were taken of the X-gal stained gross tissue, and the blue area was identified and measured using Image J software (NIH). For the microscopic examination, sections of the grossly blue area were cut to 8 μm thickness. The percentage of cells expressing β-galactosidase was determined by averaging counts of cells from 5 microscopic sections randomly selected from within the grossly stained blue region (100 cells per section, 500 cells per animal).

2.4. Statistical analysis

All parameters are summarized as mean ± S.E.M. Since means in the multiple groups were compared, an ANOVA model was performed. Since the overall p-value was less than 0.05 and the data were not balanced, Tukey's studentized range test was used to control for multiple comparisons. The corrected p-values were reported. A $p<0.05$ was considered statistically significant.

3. Results

3.1. Baseline

Our starting point was chosen to have a mid-range level of gene transfer, so that any increase or decrease in gene transfer