Efficient and Stable Transduction of Cardiomyocytes After Intramyocardial Injection or Intracoronary Perfusion With Recombinant Adeno-Associated Virus Vectors

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Background—The delivery of recombinant genes to cardiomyocytes holds promise for the treatment of a variety of cardiovascular diseases. Previous gene transfer approaches that used direct injection of plasmid DNA or replication-defective adenovirus vectors have been limited by low transduction frequencies and transient transgene expression due to immune responses, respectively. In this report, we have tested the feasibility of using intramyocardial injection or intracoronary infusions of recombinant adeno-associated virus (rAAV) vectors to program transgene expression in murine cardiomyocytes in vivo.

Methods and Results—We constructed an rAAV containing the LacZ gene under the transcriptional control of the cytomegalovirus (CMV) promoter (AAV\textsubscript{CMV-LacZ}). We then injected $1 \times 10^8$ infectious units (IU) of this virus into the left ventricular myocardium of adult CD-1 mice. Control hearts were injected with the Ad\textsubscript{CMV-LacZ} adenovirus vector. Hearts harvested 2, 4, and 8 weeks after AAV\textsubscript{CMV-LacZ} injection demonstrated stable $\beta$-galactosidase ($\beta$-gal) expression in large numbers of cardiomyocytes without evidence of myocardial inflammation or myocyte necrosis. In contrast, the Ad\textsubscript{CMV-LacZ}-injected hearts displayed transient $\beta$-gal expression, which was undetectable by 4 weeks after injection. Explanted C57BL/6 mouse hearts were also perfused via the coronary arteries with $1.5 \times 10^9$ IU of AAV\textsubscript{CMV-LacZ} and assayed 2, 4, and 8 weeks later for $\beta$-gal expression. $\beta$-Gal expression was detected in up to 50% of cardiomyocytes 4 to 8 weeks after perfusion.

Conclusions—Direct intramyocardial injection or coronary artery perfusion with rAAV vectors can be used to program stable transgene expression in cardiomyocytes in vivo. rAAV appears to represent a useful vector for the delivery of therapeutic genes to the myocardium. (Circulation. 1999;99:201-205.)

Key Words: myocardium ■ genes ■ molecular biology

Myocardial gene therapy holds promise for the treatment of a number of cardiovascular diseases, including ischemic cardiomyopathies, congestive heart failure, and malignant arrhythmias. The ideal vector for myocardial gene delivery would allow the efficient and stable transduction of cardiomyocytes with a variety of transgenes after either direct intramyocardial injection or infusion into the coronary arteries. Plasmid DNA vectors injected directly into the left ventricular myocardium are expressed for $\geq$6 months by cardiomyocytes adjacent to the area of injection. To date, however, the therapeutic usefulness of this approach has been limited by the low efficiency of cardiomyocyte transduction (0.1% to 1.0% of cardiomyocytes in the area of injection). Both intramyocardial injection and intracoronary infusion of replication-defective adenovirus (RDAd) vectors can be used to efficiently transduce cardiomyocytes in rodents, rabbits, and pigs in vivo. However, the feasibility of adenovirus-mediated gene transfer has been limited by immune responses to viral and foreign transgene proteins, which cause significant myocardial inflammation, eliminate virus-transduced cells within 30 days of infection, and thereby result in transient recombinant gene expression in immunocompetent hosts.

Recently, recombinant adeno-associated virus (rAAV) vectors have been shown to program efficient and stable recombinant gene expression in skeletal muscle and liver in both rodents and primates. Unlike RDAd, rAAV vectors do not encode viral proteins and have not been associated with immune responses to foreign transgene proteins. A previous report has shown that rAAV can transduce cardiomyocytes in vivo. However, in that study, the efficiency of rAAV-mediated transgene expression in the heart was low ($\approx$0.2%). In the present report, we have assessed the efficiency and stability of rAAV-mediated gene transfer in the heart after...
both intramyocardial injection and intracoronary infusion. Our results suggest that rAAV vectors may be useful vectors for myocardial gene delivery.

**Methods**

### Plasmids and Viruses

The structure of pAAV CMV-LacZ is shown in Figure 1A. Ad CMV-LacZ and the E3-deleted adenovirus, Ad dl309, were propagated and purified as described previously.6

Propagation and Purification of rAAV

rAAV was prepared as described.11 We determined viral titer by using a dot blot hybridization assay to determine the number of viral genomes per milliliter and by infecting HeLa cells with the virus and staining with X-gal 24 hours after infection. All viral preparations had titers of 1 to 2 \(10^{11}\) genomes/mL and 2 to 3 \(10^9\) infectious units (IU)/mL.

### In Vitro Infection of Neonatal Rat Cardiomyocytes

Cultures of primary neonatal rat cardiomyocytes 12 (4 \(10^6\) cells) were infected with 4 \(10^8\) IU of rAAV with or without 3 \(10^7\) plaque-forming units (pfu) of Ad dl309 (n = 3 for each group). After 24 to 48 hours, cells were fixed and stained with X-gal.

### Intramyocardial Injection of rAAV or RDAd

Six- to 8-week-old CD-1 mice were anesthetized, intubated, and mechanically ventilated. AAV CMV-LacZ (1 \(10^9\) IU) or Ad dl309 (2 \(10^9\) pfu) in a volume of 50 \(\mu\)L was injected into the apex of the left ventricle with a 30-gauge needle (n = 3 for each time point).

### Intracoronary Perfusion With rAAV

Adult C57BL/6 mouse hearts were perfused via the left carotid artery with cardioplegia solution (110 mmol/L NaCl, 25 mmol/L KCl, 22 mmol/L NaHCO₃, 16 mmol/L MgCl₂, 0.8 mmol/L CaCl₂, 40 mmol/L glucose) at 4°C until they stopped beating. They were then perfused ex vivo for 15 minutes with 1.5 \(10^9\) IU of AAV CMV-LacZ in 0.5 mL of PBS at a rate of 33 \(\mu\)L/min at 4°C. After perfusion, the hearts were transplanted into the neck of a syngeneic host with anastomosis of the donor aorta to the right common carotid artery of the host and anastomosis of the donor pulmonary artery to the right external jugular vein 13 (n = 3 for each time point).

### X-Gal Staining

Freshly isolated hearts were fixed in PBS plus 1.25% glutaraldehyde for 10 minutes at room temperature, stained overnight with X-gal, and counterstained with eosin.

### β-Galactosidase Activity

Cardiac homogenates were assayed for β-galactosidase (β-gal) activity and protein concentration.2 β-Gal activities were normalized for total protein and for the number of infectious rAAV or RDAd particles injected.

### Results

**Efficient In Vitro Transduction of Cardiomyocytes by rAAV Vectors**

To test the capacity of rAAV vectors to transduce cardiomyocytes in vitro, we constructed an rAAV (AAV CMV-LacZ) containing the LacZ gene under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter (Figure 1A). This virus was used to infect cultures of primary neonatal rat cardiomyocytes at a multiplicity of infection (MOI) of 100 IU/cell. Duplicate cultures were infected with AAV CMV-LacZ (MOI = 100 IU/cell) plus 2 pfu/cell Ad dl309. In other cell types, coinfecion with adenovirus has been reported to facilitate the conversion of rAAV genomes to double-stranded DNA and to thereby increase the efficiency of rAAV-mediated transgene expression.14 Twenty-four to 48 hours after infection, β-gal expression was detected by staining with X-gal. In the absence of added adenovirus, \(\approx 10\%\) of cardiomyocytes expressed β-gal (Figure 1B). The addition of adenovirus increased transduction frequencies to \(\approx 50\%\) (Figure 1C). These results demonstrate that rAAV vectors can be used to transduce primary cardiomyocytes in vitro.

**Stable Transduction of Cardiomyocytes In Vivo After Direct Intramyocardial Injection of rAAV Vectors**

To determine if rAAV could be used to stably transduce cardiomyocytes in vivo, 1 \(10^9\) IU of AAV CMV-LacZ was...
Figure 2. Gene transfer into cardiomyocytes in vivo after intramyocardial injection or coronary artery perfusion with AAV
CMV-LacZ. A, Gross sections (left) and photomicrographs (right) of mouse hearts after intramyocardial injection with $1 \times 10^9$ IU of AAV
CMV-LacZ and staining with X-gal. B, $\beta$-Gal activity in heart lysates at different times after intramyocardial injection of AAV
CMV-LacZ (n=3) or AdCMV-LacZ (n=3). Data are presented as mean±SEM. C, Gross sections (left) and photomicrographs (right) of mouse hearts after coronary artery perfusion with $1.5 \times 10^9$ IU of AAV
CMV-LacZ and staining with X-gal. Bar=25 μm.
injected directly into the left ventricular apical myocardium of 8-week-old immunocompetent CD-1 mice. Mice were killed 2, 4, and 8 weeks after injection, and β-gal expression in the myocardium was assayed by X-gal staining. As shown in Figure 2A, β-gal expression was detected in a small number of cells surrounding the site of injection at 2 weeks after injection and in a larger number of cells at 4 and 8 weeks after injection. The majority of the β-gal-positive cells were cardiomyocytes, as evidenced by their readily identifiable myofibers (Figure 2A). Furthermore, the rAAV-injected hearts did not display detectable myocardial inflammation (assessed by hematoxylin and eosin staining) or myocyte necrosis (Figure 2A and data not shown), as has been seen after myocardial injection of RDAd.3–6 To directly compare the efficiency and stability of rAAV-mediated gene transfer with those of RDAd, adult CD-1 mouse hearts were injected with either AAVCMV-LacZ or AdCMV-LacZ and quantitatively assayed for β-gal activity at different times after injection (Figure 2B). Consistent with previous reports, direct intramyocardial injection of RDAd resulted in transient transgene expression, with peak levels of β-gal activity seen 1 week after injection.3–6 By 4 weeks after injection, transgene expression was undetectable in the AdCMV-LacZ-injected hearts. In contrast, β-gal activity in the AAVCMV-LacZ-injected hearts exceeded that seen in the AdCMV-LacZ-injected hearts at both 2 and 4 weeks after injection. Peak levels of β-gal activity in the AAVCMV-LacZ-injected hearts were ≈25% of those seen with AdCMV-LacZ. Thus, rAAV vectors can be used to stably transduce cardiomyocytes in vivo with significant myocardial inflammation and with an efficiency of at least 25% relative to adeno virus vectors.

Stable and Efficient Transduction of Cardiomyocytes After Intracoronary Perfusion With rAAV

Many clinical applications of myocardial gene therapy will require the stable and efficient transduction of cardiomyocytes distributed throughout large areas of myocardium. Coronary artery infusions of RDAd have been shown to result in the efficient transduction of cardiomyocytes throughout the region of perfused myocardium.6 To test whether rAAV is similarly capable of transducing cardiomyocytes after coronary artery perfusion, we explanted hearts from C57BL/6 mice and perfused them with 1.5 × 109 IU of AAVCMV-LacZ for 15 minutes at 4°C via a catheter placed in the left common carotid artery. These perfused hearts were then transplanted into syngeneic hosts, and the arterial circulation was reestablished by anastomosis of the transplanted aorta to the recipient carotid artery. Such transplanted and revascularized hearts resumed beating and continued to do so until the recipient mice were killed 2, 4, or 8 weeks after perfusion. Two weeks after perfusion, small numbers (<1%) of β-gal–positive cardiomyocytes were detected throughout the myocardium of the rAAV-perfused hearts (Figure 2C). By 4 weeks after perfusion, ≈40% of the cardiomyocytes were β-gal positive. This high level of transduction was stable at 8 weeks after perfusion, with >50% of the cardiomyocytes continuing to express β-gal. Similar increases in recombinant gene expression over the first several weeks after rAAV infection have been observed in skeletal muscle.7,8 It has been postulated that such increases may reflect the gradual process of conversion of the single-stranded AAV genome into a double-stranded DNA molecule that is competent for transcription of the transgene.14 Thus, rAAV delivered by coronary artery perfusion can be used to stably transduce cardiomyocytes throughout the myocardium.

Discussion

In this report, we have demonstrated efficient and stable transduction of cardiac myocytes in vivo after intramyocardial injection or intracoronary infusions of rAAV vectors. Our results suggest that rAAV displays significant advantages for myocardial gene transfer compared with either plasmid DNA or adenovirus vectors. Plasmid DNA vectors produce low-efficiency (albeit stable) cardiomyocyte transduction and can only be administered by direct intramyocardial injection.2–5 In contrast, rAAV allows efficient transduction of cardiomyocytes and can be administered by either intramyocardial injection or intracoronary infusion. Adenovirus vectors allow highly efficient cardiomyocyte gene transfer but produce intense intramyocardial inflammation and myocyte necrosis, thereby resulting in transient recombinant gene expression in immunocompetent hosts in vivo.3–6 In contrast, rAAV vectors program stable expression of foreign transgenes in immunocompetent hosts. The stability of transgene expression observed with rAAV even after expression of a foreign transgene protein likely reflects the fact that rAAV vectors, unlike their adenovirus counterparts, do not express any viral gene products and are therefore significantly less immunogenic. This lack of immunogenicity represents a major advantage of rAAV for myocardial gene transfer.

Despite these advantages, there are several issues that might limit the utility of rAAV for cardiovascular gene therapy. First, this vector can only accept transgenes less than ≈4.5 kb in length. Second, current techniques do not allow the convenient production of large amounts of rAAV. Finally, although our studies have demonstrated efficient transduction of cardiomyocytes after 15 minutes of coronary artery perfusion with rAAV, it remains unclear if similar high-efficiency transduction can be obtained by catheter-mediated intracoronary infusions of this vector. Despite these caveats, our results suggest that rAAV vectors will significantly enhance our ability to stably express recombinant genes in cardiomyocytes in vivo and, as such, may represent an important vector system for myocardial gene therapy.

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