Expression of Recombinant Genes in Myocardium In Vivo After Direct Injection of DNA

Hua Lin, MD, Michael S. Parmacek, MD, Gerald Morle, BS, Steven Bolling, MD, and Jeffrey M. Leiden, MD, PhD

The ability to program recombinant gene expression in cardiac myocytes in vivo holds promise for the treatment of many inherited and acquired cardiovascular diseases. In this report, we demonstrate that a recombinant β-galactosidase gene under the control of the Rous sarcoma virus promoter can be introduced into and expressed in adult rat cardiac myocytes in vivo by the injection of purified plasmid DNA directly into the left ventricular wall. Cardiac myocytes expressing recombinant β-galactosidase were detected histochemically in rat hearts for at least 4 weeks after injection of the β-galactosidase gene. These results demonstrate the potential of this method of somatic gene therapy for the treatment of cardiovascular disease. (Circulation 1990;82:2217–2221)

Somatic gene therapy, the expression of recombinant genes in non-germ-line tissues of the adult organism, holds great promise for the treatment of many inherited and acquired human diseases (reviewed in Reference 1). The biological requirements for this type of gene therapy include the ability to introduce recombinant genes efficiently into the appropriate cells and tissues and to program the high-level and, in many cases, stable expression of these recombinant genes in vivo. In addition, it is necessary that the process of gene therapy itself not be harmful to the recipient organism, in particular, that the techniques used to introduce the recombinant genes do not result in persistent infection of the host or in deleterious mutations of the recipient cells. Two general approaches have proven useful in animal models of somatic gene therapy. In the first, recombinant genes have been introduced into cultured cells in vitro, and cells expressing the recombinant gene product have then been transplanted into the appropriate tissue of a recipient animal.2–4 In the second, recombinant genes have been introduced directly into somatic cells in vivo.5

The ability to program recombinant gene expression in adult myocardium in vivo requires both an expression vector with high-level activity in cardiac myocytes and a method for introducing such a vector into myocardiual cells in the adult animal. A previous study demonstrated that murine skeletal myocytes display a rather unique ability to take up and express DNA after direct injection in vivo.6 In the studies described in this report, we show that an expression vector using the Rous sarcoma virus (RSV) long terminal repeat (LTR) programs high-level recombinant gene expression in rat cardiac myocytes in vitro and demonstrate that recombinant genes cloned into this vector can be introduced into and expressed in adult rat cardiac myocytes for at least 4 weeks after direct injection of plasmid DNA into the left ventricular wall.

Methods

Cell Culture and Transient Transfections

Neonatal rat cardiac myocytes were isolated from 1–2-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) by collagenase digestion as previously described.7 This method results in the isolation of more than 90% cardiac myocytes.7 Twenty-four hours after isolation, $1 \times 10^6$ freshly isolated myocytes in a 60-mm collagen-coated dish (Collaborative Research Inc., Waltham, Mass.) were transfected with 15 μg of cesium chloride gradient-purified chloramphenicol acetyl transferase (CAT) reporter plasmid DNA plus 5 μg of pMSVβgal reference plasmid DNA as follows: 20 μg of plasmid DNA was resuspended in 1.5 ml of Opti-MEM (GIBCO, Grand Island, N.Y.)
Figure 1. Transcriptional activity of the Rous sarcoma virus (RSV) long terminal repeat (LTR) in rat neonatal cardiocytes in vitro. Panel A: A schematic representation of the pRSVCAT and pRSVβgal plasmids. βgal, β-galactosidase gene; CAT, chloramphenicol acetyl transferase gene. HindIII (H3) and BamHI (B) restriction endonuclease sites are shown. Panel B: Transcriptional activity of the RSV LTR in rat neonatal cardiocytes in vitro. Rat neonatal cardiocytes were transfected with 15 μg of the promoterless pSVOCAT control plasmid or the pRSVCAT plasmid (see panel A) and cell extracts prepared 48 hours after transfection were normalized for protein content and assayed for CAT activity as previously described. To control for differences in transfection efficiencies, all transfections also contained 5 μg of the pMSVβgal reference plasmid. Data are shown as CAT activity relative to that produced by the pSVOCAT plasmid (which produced 1.7% acetylation) after correction for differences in transfection efficiency.

and added to 1.5 ml of Opti-MEM containing 50 μl of lipofectin reagent (BRL, Gaithersburg, Md.). The resulting mixture was added to one 60-mm plate of cardiac myocytes. After 5 hours at 37°C in 5% CO₂, 3 ml of Medium 199 plus 5% fetal bovine serum (FCS) (GIBCO) was added to the cells, and the mixture was incubated at 37°C for 48 hours. Cell extracts were prepared and normalized for protein content using a commercially available kit (Biorad, Richmond, Calif.). CAT and β-galactosidase assays were performed as previously described.

Plasmids

The promoterless pSVOCAT plasmid and the pRSVCAT plasmid in which transcription of the bacterial CAT gene is under the control of the RSV promoter have been described previously. The pRSVβgal plasmid was constructed by cloning the 4.0-kb β-galactosidase gene from pMSVβgal into HindIII/BamHI-digested pRSVCAT (see Figure 1A).

Injection of Recombinant DNA In Vivo

Six- to 11-week-old 250-g Sprague-Dawley rats were housed and cared for according to National Institutes of Health guidelines in the ULAM facility of the University of Michigan Medical Center. Rats were anesthetized with 20 mg/kg pentobarbital i.p. and 60 mg/kg ketamine i.m., intubated, and ventilated with a Harvard (Harvard Apparatus, South Natick, Mass.) respirator. A left lateral thoracotomy was performed to expose the beating heart, and 100 μg of plasmid DNA in 100 μl of phosphate-buffered saline (PBS) containing 5% sucrose (PBS/sucrose) was injected into the apical portion of the beating left ventricle using a 30-g needle. Control animals were injected with 100 μl of PBS/sucrose alone. The animals were killed 3–5 or 21–30 days after injection by pentobarbital euthanasia; hearts were removed via a median sternotomy, rinsed in ice-cold PBS, and processed for β-galactosidase activity.

Histochemical Analysis

Three-millimeter cross sections of the left ventricle were fixed for 5 minutes at room temperature with 1.25% glutaraldehyde in PBS, washed three times at room temperature in PBS, and stained for β-galactosidase activity with X-gal (Biorad) for 4–16 hours as described by Nabel et al. The 3-mm sections were embedded with glycomethocrylate, and 4–7-μm sections were cut and counterstained with hematoxylin and eosin as described previously. Photomicroscopy was performed using Kodak Ektachrome 200 film and Leitz Laborlux D and Wild M8 microscopes.
Results

RSV LTR Promotes High-Level Gene Expression in Rat Neonatal Cardiocytes In Vitro

Although the RSV LTR displays high-level transcriptional promoter activity in a wide variety of immortalized cell types,11 previous transgenic studies have suggested that this promoter is preferentially active in skeletal and cardiac myocytes in vivo.13,14 To test directly the transcriptional activity of the RSV LTR in rodent cardiac myocytes, the pRSVCAT vector11 in which expression of the bacterial CAT gene is under the control of the RSV LTR was transfected into primary neonatal rat cardiac myocytes using lipofectin. Two days after transfection, the cultures were harvested and assayed for CAT activity as previously described.9 All transfections also contained 5 μg of the pMSVβgal plasmid12 to correct for differences in transfection efficiencies. As shown in Figure 1, the RSV LTR was able to increase transcription of the CAT gene 87-fold compared with the promoterless pSVOCAT control plasmid. The pRSVCAT-transfected cardiac myocyte extracts produced 95% acetylation in a standard thin-layer chromatography assay.9 By comparison, identically prepared extracts of 3T3 or HeLa cells transfected with this same vector produced 22% and 35% acetylation, respectively (data not shown). Because the activities of cotransfected pMSVβgal reference plasmids were almost identical in all three transfections, these results demonstrated that the RSV LTR programs high-level transcription in primary cardiac myocytes in vitro.

The ability to unambiguously identify the cell types that are expressing recombinant gene products is an important requirement of all animal models of gene therapy. Because the bacterial β-galactosidase reporter gene (but not the bacterial CAT gene) allows direct histological visualization of recombinant gene expression, we constructed a pRSVβgal vector in which bacterial β-galactosidase gene expression is regulated by the RSV LTR promoter for further studies of recombinant gene expression in vivo (Figure 1B).

Expression of β-Galactosidase Gene in Rat Cardiac Myocytes After Injection of pRSVβgal DNA Into the Left Ventricular Wall In Vivo

In an attempt to program recombinant β-galactosidase gene expression in rat cardiac myocytes in vivo,
vivo, we took advantage of a previously described technique for producing recombinant gene expression in murine skeletal myocytes in vivo. Briefly, 100 μg of pRSVβgal DNA was resuspended in 100 μl of PBS containing 5% sucrose (PBS/sucrose) and injected via a 30-g needle directly into the beating left ventricular wall of 6-11-week-old Sprague-Dawley rat hearts. Control rats received injections of 100 μl of PBS/sucrose without DNA. Rats were killed either 3-5 days or 3-4 weeks after injection, and hearts were fixed and stained for β-galactosidase activity. β-Galactosidase activity as manifested by dark-blue staining was readily apparent to the naked eye in sections of three of four of the pRSVβgal-injected hearts at 3-5 days and four of five of the pRSVβgal-injected hearts at 3-4 weeks after DNA injections (Figures 2A and 2B). This staining, which was focal and patchy, occurred only in a single area of each heart injected with pRSVβgal DNA and was not seen in five control hearts injected with PBS/saline alone (data not shown). Failure to observe staining in two of nine of the pRSVβgal-injected hearts may have been due to the lack of DNA uptake or expression in these hearts or, more likely, to technical difficulties in successfully centering and anchoring the needle in the relatively thin beating left ventricular wall during the injection process.

Because the normal ventricular wall contains both myocytes and fibroblasts and because the injection of DNA might be expected to cause a localized inflammatory response, it was important to determine which cell types were expressing the recombinant β-galactosidase gene. Histochemical analysis of sections from hearts injected with the pRSVβgal DNA clearly demonstrated β-galactosidase activity within cardiac myocytes that were easily identified by their myofibrillar architecture (Figures 2C and 2D). Between one and 10 positively staining myocytes were seen per high-power field, and these were often noncontiguous, suggesting that the uptake of DNA and/or its expression is a relatively low-frequency event. Because it was difficult to accurately identify the extent of DNA injection and because the positively staining areas were quite focal and patchy, it was impossible to accurately quantitate either the percentage or the total number of cells expressing recombinant β-galactosidase activity in a given heart. However, it is clear that only a small fraction of cardiac myocytes expressed the recombinant protein. In addition, it is worth noting that sections from the 3-5-day postinjection hearts often showed evidence of an acute inflammatory response along the track of the needle (Figure 2C) and that in several cases fibrosis along the needle track was observed in sections from the 3-4-week postinjection hearts (data not shown).

Discussion

The studies presented have demonstrated that it is possible to program recombinant gene expression in cardiac myocytes after direct injection of DNA into the left ventricular wall. Functional recombinant protein expression in myocytes was demonstrated directly using an enzymatic assay for β-galactosidase. Recombinant gene expression was observed in myocytes from seven of nine of the injected hearts at both 3-5 days and 3-4 weeks after injection. Expression was patchy and was observed only in direct contiguity with the site of injection. These findings have several implications regarding both the use of this method for somatic gene therapy in the heart and the biology of recombinant DNA uptake and expression in muscle cells.

A previous study suggested that murine skeletal muscle cells possess a unique ability to take up and express injected recombinant DNA. Our results have extended this observation to cardiac muscle cells in a second rodent species. It has previously been thought that successful DNA transfection and expression may require recipient cell division and, more specifically, breakdown of the recipient cell nuclear membrane to allow DNA entry. Because skeletal myocytes have a limited potential for mitosis, it remained possible that the previously reported successful transfection of skeletal myocytes was dependent on their mitotic potential. In contrast to skeletal myocytes, adult cardiac muscle cells are unable to divide. Thus, our results demonstrate that mitosis is not necessary for successful transfection of cells with DNA. The mechanisms that allow preferential uptake of DNA into cardiac and skeletal myocytes remain unclear. However, our data suggest that they must be dependent on structural or functional properties that are shared by skeletal and cardiac muscle. Current hypotheses include the possibility of specialized muscle cell transport systems or the unique ability to physically disrupt the cell membranes of muscle cells in a reversible fashion during the recombinant DNA injections.

The technique of somatic gene therapy using direct DNA injection into myocardium, as described in this report, has several advantages compared with other previously described methods of gene therapy. First, infectious viral vectors are not required, eliminating the possibility of persistent infection of the host. Second, a previous study has suggested that recombinant DNA taken up and expressed in skeletal myocytes persists as an episome and therefore does not have the same potential for host cell mutagenesis as do retroviral vectors that integrate into the host chromosome. Finally, this method does not require the growth of recipient cells in vitro, a requirement that would render transfection of nondividing cardiac myocytes particularly difficult.

Direct injection of recombinant DNA into the myocardium holds promise for the treatment of many acquired and inherited cardiovascular diseases. We are particularly interested in the possibility of stimulating collateral circulation in areas of chronic myocardial ischemia by expressing recombinant angiogenesis factors locally in the ventricular wall. Although the method described in this report is a first step toward such gene therapy approaches, many questions and problems remain to be addressed.
before this type of gene therapy can become a reality. First, it must be demonstrated that human myocytes, like their rodent counterparts, are able to take up and express recombinant DNA. The longevity of recombinant gene expression must be more fully examined, and the possibility that some of the recombinant DNA is integrated into the host genome with the concomitant potential for mutagenesis must be ruled out. Modifications of the current transfection protocol must be developed to increase the frequency of recombinant gene expression in cardiac myocytes. Of equal importance, the inflammatory response to the injected DNA must be controlled to prevent the formation of arrhythmogenic foci. Finally, it will of interest to determine whether high-level recombinant gene expression can be programmed in vivo by the injection of expression vectors containing cardiac-specific transcriptional regulatory elements. Ongoing studies in our laboratory are designed to address these problems. Nevertheless, the initial studies described in this report suggest that somatic gene therapy in the heart may eventually become a useful therapeutic modality.

Acknowledgments

The authors would like to thank Dr. James Wilson for critical review of the manuscript, Mrs. Jeanelle Pickett for expert secretarial assistance, and Ms. Beverly Burck for the preparation of the illustrations. We would also like to thank Kaye Brabec, Lorita Dudus, and Dr. Kent Christensen for help with histochemical staining.

References


KEY WORDS • gene therapy • DNA
Expression of recombinant genes in myocardium in vivo after direct injection of DNA.
H Lin, M S Parmacek, G Morle, S Bolling and J M Leiden

*Circulation*. 1990;82:2217-2221
doi: 10.1161/01.CIR.82.6.2217

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/82/6/2217

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/