Foreign Gene Transfer to Cardiomyocyte Using a Replication-Defective Recombinant Coxsackievirus B3 without Cytotoxicity

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Key Words
Replication-defective recombinant coxsackievirus • Transfer vector • Cardiac myocytes • Cardiotropic virus • Cytotoxicity

Abstract
Background: Replication-competent coxsackievirus B3 (CVB3) has been used as a gene transfer vector for cultured cardiomyocytes and hearts in vivo. However, CVB3 induces cell lysis when it replicates in infected cells. In this study, we investigated whether a replication-defective rCVB3 vector could be generated and used as a noncytotoxic gene transfer vector for cardiomyocytes. Methods: We generated a replication-defective luciferase-expressing CVB3 plasmid. This recombinant cDNA and pCMV-P1 plasmids were amplified and cotransfected into Hek293 cells using transfection reagents. Replication-defective rLuCVB3 virus was recovered from the cells and cell culture supernatants for 3 days after transfection. The generated rLuCVB3 viruses were concentrated on a 30% sucrose cushion and semiquantified using a luciferase assay. In addition, foreign gene delivery by the rLuCVB3 was tested in cultured cardiomyocytes and intact mouse hearts after rLuCVB3 infection. Results: Luciferase was expressed in Hek293, HeLa cells and cardiomyocytes after rLuCVB3 infection. In addition, these cells did not show a significant cytopathic effect after 72 h. Luciferase protein expression or activity were detected for 3 days in the myocardium of rLuCVB3-infected mouse hearts without producing cytotoxicity or inflammation. Conclusion: As a proof-of-concept, these data indicate that a replication-defective rCVB3 vector can be generated and used as a novel gene transfer system to transfect exogenous genes into cardiomyocytes without generating cytotoxicity.

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Introduction
Coxsackievirus B3 (CVB3) is a member of the picornaviridae family of enteroviruses. It has a 7.4-kb positive single-strand RNA genome that is translated as a monocistronic polyprotein [1, 2]. The CVB3 genome encodes capsid proteins (P1: VP4, VP2, VP3 and VP1) and nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) that are involved in viral replication [3]. Nonstructural proteins 2A and 3C are cotranslationally cleaved into mature peptides. CVB3 is a highly cardiotropic virus and the most common pathogen that causes human viral myocarditis. CVB3 requires the coxsackie and adenovirus receptor (CAR) to attach and penetrate the target host cells.

B.-K.L. and S.-H.Y. contributed equally to this project.
CAR is expressed at very low levels in the normal adult heart, but it is highly expressed during cardiomyopathy or in a damaged heart. Therefore, CVB3 might potentially be useful as a vector that would allow the expression of exogenous proteins in the heart. Our previous studies have shown that replication-competent CVB3 vectors can be generated and used to facilitate gene transfer into neonatal and adult myocytes in culture and into adult mouse hearts in vivo [4]. However, this recombinant CVB3 vector elicits an immune response and causes tissue toxicity, which is a crucial drawback for a viral vector system. Coxsackievirus is typically considered a lytic virus that activates a potent immune response in the heart [5–7], but the recombinant CVB3 induces sparse inflammation in the heart and little myocardial injury [4]. Therefore, we investigated whether a replication-defective recombinant CVB3 could be engineered to express exogenous proteins in cardiomyocytes without cytotoxicity. Previous studies have explored the use of modified polioviruses as recombinant vectors to deliver genes into cells for the purpose of vaccination. A recombinant poliovirus was constructed in which a reporter gene was substituted for the P1 capsid gene and the deleted capsid liovirus was constructed in which a reporter gene was expressed by vaccinia P1 virus for recombinant exogenous proteins in cardiomyocytes without cytotoxicity. These rCVB3 vectors might prove potential therapeutic tools to deliver exogenous proteins for treating human cardiovascular diseases.

**Materials and Methods**

**Cell Culture**

Human embryonic kidney (Hek) 293 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS; Invitrogen Co., Carlsbad, Calif., USA) and Chinese hamster ovary (CHO) cells were maintained in RPMI 1640 (Invitrogen) with 10% FBS, under standard cell culture conditions at 37°C and 5% CO₂ in air. Hek293 cells were used for virus production because of their good transfection efficiency. Primary rat neonatal cardiomyocytes were isolated from the hearts of neonatal Sprague-Dawley rats less than 3 days old, and cultured as described [4].

**Cloning Recombinant Viral cDNA and Capsid Protein Expression Vectors**

To test the application of rCVB3 as a nontoxic cardiotropic gene transfer vector, we used as the starting point GFP-CVB3 cDNA in pBluescript, as described previously [4], which contains the entire full-length CVB3 infectious cDNA and the sequence of an enhanced reporter gene for green fluorescent protein (GFP). The GFP-CVB3 sequence includes an in-frame synthetic polylinker containing the EcoRI and XhoI sites into which is inserted the GFP cDNA. The first six amino acids (MAAQEF) of the CVB3 VP4 capsid protein were inserted at the amino terminus of the GFP sequence. The luciferase (Lu) sequence was amplified by polymerase chain reaction (PCR) from the pGL3-Basic plasmid (Promega, Madison, Wisc., USA), and used to substitute the GFP-P1 capsid protein coding sequences in GFP-CVB3 [7] (pLuCVB3) (fig. 1A). For producing recombinant CVB3, the deleted CVB3 capsid P1 sequence was amplified by PCR and subcloned into the mammalian expression plasmid pcDNA3.1 (Invitrogen) using unique restriction enzyme sites (pCMV-P1) (fig. 1B). We used techniques similar to those that have been used to express exogenous antigens in a poliovirus vector system [12, 13].

**Replication-Defective Recombinant LuCVB3 (rLuCVB3)**

To generate the rLuCVB3 viral vector, the pLuCVB3 and pCMV-P1 plasmids were amplified and cotransfected into Hek293 cells using FuGENE 6 (Roche, Mannheim, Germany). The rLuCVB3 was recovered from the cell and culture supernatants for 3 days after transfection. The cells were broken down by double freeze/thaw cycle with subsequent centrifugation at 9,300 g for 30 min and filtration through a 0.22-μM syringe filter [14]. The cleared extract was collected and stored at –70°C before use. Generated rLuCVB3 was confirmed by luciferase activity in infected Hek293 cells. Hek293 cells were seeded at 10⁵ cells/well in 96-well plates and cultured for 24 h; then rLuCVB3 supernatant was added to the wells and incubated for 24 h more. Luciferase expression was estimated using a firefly luciferase assay kit (Promega) and a single-tube TD20/20 luminometer (Turner Designs, Sunnyvale, Calif., USA). A cutoff point of 10-fold induction of chemiluminescence relative to that of the uninfected control cells (set at 1) after normalization to the fluorescence of the uninfected control proteins (RLU/mg) was used to determine if the cells in a well had been infected successfully.

**Concentration of rLuCVB3**

The rLuCVB3 virus vector was concentrated using a sucrose cushion method [15]. Cotransfection supernatants were centrifuged in a Beckman ultracentrifuge (XL-90 with an SW28 rotor) at 82,740 g for 30 min at 8°C. The resulting supernatant was layered onto a 30% (w/v) sucrose cushion (in 10 mM Tris pH 7.5, 100 mM NaCl) and ultracentrifuged using a SW-28 swing rotor for 18 h at 141,000 g for 30 min and filtration through a 0.22-μM syringe filter. The rLuCVB3 pellet was resuspended in phosphate-buffered saline and stored at –70°C.

**Estimation of the Titer of the Replication-Defective rLuCVB3**

The rLuCVB3 vector has the capacity to infect cells but it cannot replicate and form plaques, so the viral titers cannot be quantified by traditional plaque-forming unit (PFU) assays. To overcome this problem, we used immunofluorescent staining for luciferase to estimate the titer of rLuCVB3 by comparison with the known titer of wild-type CVB3 [12]. The resulting titer was then expressed as infectious units. We determined that the infectious unit value of the replication-defective virus correlated significantly with the PFU of wild-type coxsackievirus.
Target Cell Infection and Measurement of Cytotoxicity

Hek293, HeLa, CHO cells and cardiomyocytes (10^3 cells) were seeded into the wells of 96-well plates using the appropriate medium as described above and cultured for 24 h. rLuCVB3 was added into the wells at a multiplicity of infection (MOI) of 10 and incubated for a further 1 h, after which the medium was changed to medium containing 1% FBS and the cells were cultured until assayed for luciferase activity and cytotoxicity. At 0, 2, 4, 8, 16, 24, 48 and 72 h postinfection (pi), the culture media were collected for the measurement of cytotoxicity using lactate dehydrogenase (LDH) assay kits (Roche). The cells were lysed directly in the culture wells and assayed with luciferase assay kits (Promega) according to the manufacturer’s instructions.

Western Blot Analysis

Each cell was lysed in sodium dodecyl sulfate (SDS) sample buffer (25 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol and 0.1% w/v bromophenol blue). Aliquots of 10 μg of total cell extracts were loaded onto 10% SDS-polyacrylamide electrophoresis (PAGE) gels and transferred to Hybond-ECL nitrocellulose membrane (Amersham, Bucks., UK). The membranes were blocked in 5% nonfat dry milk solution (in Tris-buffered saline) containing 0.1% Tween-20. Protein bands were probed with anti-luciferase (NEB, Beverly, Calif., USA) and anti-tubulin (NEB) antibodies followed by labeling with a horseradish peroxidase-conjugated second antibody (Jackson, Baltimore, Md., USA). Bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham).

Immunohistochemistry

Immunohistochemical analysis of myocardial tissue sections was performed as described [16] using purified mouse anti-dystrophin IgG (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and anti-luciferase (NEB). The avidin/biotin alkaline-phosphatase system (Vectorstain kit; Vector Laboratories, Burlingame, Calif., USA) was used for signal amplification. The slides
were sequentially treated with 3,3′-diaminobenzidine (DAB) Substrate Solution (Dako, Glostrup, Denmark) to develop the color reaction. Serial sections were stained with HE. Negative control staining was performed with blocking buffer instead of primary antibody.

Statistical Analysis

Data are presented as the mean ± SEM. Student’s t test was used for the analysis of numeric parameters (SPSS 10.0 for Windows; SPSS Inc., Chicago, Ill., USA). Differences were considered significant at \( p < 0.05 \).

Results

Generation of Replication-Defective Coxsackievirus B3 Vector

To investigate nontoxic cardiac-specific gene delivery, we modified a CVB3 cDNA, which is a very well-defined highly cardiotropic enterovirus. To generate a replication-defective CVB3, we cloned two different cDNA plasmids. One was a CVB3 backbone cDNA plasmid that replaced the capsid protein (P1: VP4, 2, 3 and 1) and carried a luciferase reporter gene for easy detection of virus gene delivery and for control of viral self-replication (pLuCVB3) (fig. 1A). The other was a virus capsid protein P1-producing plasmid with a mammalian CMV promoter to supply the deleted P1 protein (pCMV-P1) (fig. 1B). The infectious recombinant CVB3 virus (rLuCVB3) was recovered from cotransfection supernatants of both virus backbone and P1-expressing plasmids. After 3 days of cotransfection, Hek293 cells showed cytopathic effects indicating recombinant virus production.

Recombinant CVB3 (rLuCVB3) Expresses Luciferase in Infected Cells without Producing Cell Cytotoxicity

We examined rLuCVB3 infection and cell cytotoxicity on HeLa cell lines incubated with different volumes of rLuCVB3-containing cell supernatants. At 48 h after incubation, there was no decrease in cell viability compared with normal cell supernatants. Luciferase activity was also increased by the volume of cell supernatant. Aliquots of 1 \( \mu l \) of cell supernatant produced 100 RLU/mg protein and 100 \( \mu l \) resulted in 30,000 RLU/mg protein (fig. 2). These results suggest that rLuCVB3 could deliver the reporter gene to HeLa cells without causing cell cytotoxicity. The assumed presence of rLuCVB3 was also tested for its ability to affect cell proliferation. Fresh Hek293 cells were inoculated with supernatants that had been collected from Hek293 cell supernatants at 48 h pi with rLuCVB3. However, the luciferase activity in these cells remained negative, confirming the absence of progeny virus (data not shown).

We also studied the optimal structure of the capsid protein deletion for efficient production of rLuCVB3. It was confirmed by luciferase activity from HeLa

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Results are represented as plaque-forming units (PFU/ml) and viral titers were determined by semiquantitation of luciferase immunofluorescent staining in HeLa cell monolayers. Values are means from independent experiments, in each of which titrations were carried out in triplicate.
Foreign Gene Transfer to Cardiomyocyte without Cytotoxicity

**Fig. 3.** Comparative infection of different cells with rLuCVB3 vector to investigate whether a replication-defective rCVB3 vectors could deliver foreign proteins into cells susceptible to wild-type coxsackievirus infections. Hek293T, HeLa, rat neonatal cardiomyocytes and CHO cells were infected with LuCVB3 carrying genomes in which the P1 region was replaced by a luciferase reporter gene (MOI = 10). Luciferase activity was measured at given time points postinfection. Representative data from four experiments are presented as the mean ± SEM.

**Fig. 4.** Cytotoxicity of rLuCVB3 was tested on infected cardiomyocytes. The rLuCVB3-infected cell culture media supernatants were collected at given time points postinfection for measuring cytotoxicity using an assay of LDH activity. The LDH levels of wild-type CVB3 virus-infected cardiomyocytes increased at 16 h (4.94 ± 0.03) and peaked at 72 h (24.82 ± 0.04). However, the LDH level in rLuCVB3-infected cardiomyocytes was not significantly increased (3.04 ± 0.27), which was similar to the virus uninfected control cells (2.8 ± 0.02). Data are represented as means ± SEM of the LDH fold changes from four experiment results. Each LDH fold change was compared with the 0 h baseline expressed as 1.

**Fig. 5.** Detection of foreign gene transfer in rLuCVB3-infected intact mouse hearts. Luciferase transfer was tested in the heart after a tail vein injection of 10 MOI of rLuCVB3. **A** Luciferase activity was detected from the hearts at days 1 and 3 (n = 3 for each time point). However, the enzyme activity was quickly eliminated after expression. **B** Protein was detected by Western blot analysis from three individual mice hearts. There were no significant decreases in the amount of luciferase protein detected. Tubulin was used as an internal control indicating the total protein amount in each sample.
cells exposed to cotransfected cell supernatants. The pLuCVB3ΔVP0, pLuCVB3ΔVP3 and pLuCVB3ΔVP1 structures did not produce significant amounts of recombinant CVB3. In contrast, the pLuCVB3ΔP1 (pLuCVB3) structure allowed the efficient production of rLuCVB3, with viral stocks of approximately $10^6$ PFU/ml obtained (table 1). We used this structure to generate rLuCVB3 in our subsequent experiments.

**rLuCVB3 Delivers Reporter Protein to Cultured Rat Neonatal Cardiomyocytes**

Next, we tested the infectivity of rLuCVB3 from various cell lines to investigate the delivery of a foreign protein to cells susceptible to wild-type CVB3. The susceptible cells – Hek293, HeLa and rat neonatal myocytes – and unsusceptible CHO cells were infected with 10 MOI of rLuCVB3. Cell lysates were then collected at 1, 2, 4, 8, 16, 24 and 48 h pi. Luciferase activity was detected at $10^5$ log RLU/mg protein in Hek293 and HeLa cells and at $10^2$ log RLU/mg protein in cardiomyocytes at 16 h pi. All cell lines showed similar profiles of luciferase expression over 24 h, although the expression level varied according to the cell infectivity. In particular, luciferase was constantly expressed over 48 h in cardiomyocytes. Luciferase activity was not detected in the CHO cells (fig. 3).

**rLuCVB3 Does Not Induce Cell Cytotoxicity in Cultured Neonatal Rat Cardiomyocytes**

We found that rLuCVB3 could deliver a foreign protein to cardiomyocytes for 48 h and that there was no significant cytotoxicity in HeLa cells. Therefore, we investigated whether rLuCVB3 would induce cytotoxicity in cultured cardiomyocytes. To analyze the cytotoxicity of rLuCVB3, we measured LDH in the culture medium until 72 h pi. The LDH level in the culture medium of wild-type CVB3 increased gradually up to 72 h pi (30.8 ± 0.04-fold). However, rLuCVB3 produced no dramatic increase in LDH for this time (2.5 ± 0.27-fold), similar to uninfected control cells (2.0 ± 0.02-fold) (fig. 4). These results demonstrated that the recombinant CVB3 could deliver foreign proteins to cultured cardiomyocytes without cytotoxicity.

**rLuCVB3 Delivers Reporter Protein to the Intact Myocardium in the Adult Mouse**

To investigate whether rLuCVB3 delivers enough foreign protein to intact myocardium, mice were infected with $10^5$ PFU rLuCVB3 through a tail vein injection. Mice were euthanized and the heart was collected at 1, 3 and 7 days pi to test luciferase expression and check on cardiac pathology. Luciferase activity was detected at

![Fig. 6. rLuCVB3 can deliver luciferase into the myocardium without inflammation.](image-url)
days 1 and 3 but was soon eliminated (fig. 5A). However, luciferase protein was detected at day 3 pi by Western blotting from three different hearts (fig. 5B). In addition, we examined the presence of luciferase in an individual myocardium. Luciferase was detected using immunohistochemistry in the myocardium at day 3 pi (fig. 6A). Wild-type CVB3 is well known as a cause of viral myocarditis. Our results showed that rLuCVB3 infection did not induce inflammation in the mouse heart up to day 7 pi (fig. 6Bb, c), but CVB3 infection induced strong cardiomyocyte damage and inflammation in the heart (fig. 6Bd, e). In addition, the rLuCVB3-infected heart tissue did not exhibit dystrophin disruption (fig. 6Bg), as in control uninfected heart tissues (fig. 6Bf), but dystrophin disruption was noted following CVB3 infection (fig. 6Bh). These results demonstrated that rLuCVB3 could be used as a foreign protein delivery system into the heart without causing cardiomyocyte damage or inflammation.

**Fig. 6.** rLuCVB3 can deliver luciferase into the myocardium without inflammation. B rLuCVB3-induced cardiomyocyte damage was examined by histopathology with HE staining at days 3 and 7 pi. rLuCVB3 infection did not induce any severe inflammation or cell death (b, c). In contrast, wild-type CVB3 induced inflammation on days 3 and 7 pi (d, e). Dystrophin was not cleaved in the rLuCVB3-infected heart (g), but was cleaved in the CVB3-infected heart (h). Arrows indicate the dystrophin cleavage area (nonstained). Scale bar = 100 μm.
Discussion

In this report, we have described a novel system for the generation of replication-defective rCVB3, which was developed for myocardial gene delivery without producing cytotoxicity. Previous experiments with poliovirus and CVB3 have demonstrated that picornaviruses can be used to express foreign polypeptides or antigens [13,17–19]. We have also demonstrated that the coxsackievirus vector rCVB3-GFP can be used for long-term cardiac gene expression [4]. This rCVB3-GFP virus is a replication-competent virus vector and the immune response and tissue toxicity are crucial issues, as in any viral vector system. Although coxsackievirus is typically considered a lytic virus that activates a potent immune response in the heart [5–7], the rCVB3-GFP virus induced only sparse inflammation in the heart and little myocardial injury in a cardiac troponin T assay. The cytopathic effect is an important element in any viral vector system. Recent studies in our and other laboratories have suggested that a replication-defective coxsackievirus vector system could be used to deliver foreign proteins to cardiomyocytes without cytotoxicity [4]. In particular, studies on polioviruses have reported recombinant virus models [8–10,12]. The relative tissue tropism of CVB3 is one of its significant differences from other viral vectors, such as adenoviruses, adeno-associated virus and retroviruses. Certain variants of CVB3, such as CVB3-H3, have been selected for their increased tropism for the heart by passage in mouse hearts [7,20].

We generated three different systems, such as adenovirus vector, transgenic cell lines and mammalian expression plasmids, which expressed a single virus capsid protein such as VP0, VP3, VP1 or P1 region. Using cDNA in which each capsid protein region was deleted, we generated many different replication-defective rCVB3s. Initial tests showed that a recombinant CVB3 virus could be generated by the cotransfection of those plasmids: one allowing the transcription of the P1-deleted viral cDNA and the other permitting the expression of P1 as a polyprotein. However, a single capsid protein deletion could not generate the rCVB3 virus. When the deleted capsid protein was supplied by an adenovirus vector (Ad-P1) or a transgenic cell line (293-P1) transfected with a replicon of the rCVB3 cDNA plasmid, there was no significant production of recombinant virus particles. Therefore, we used a plasmid cotransfection system to generate the replication-defective rCVB3 vector for further studies.

Previous studies have demonstrated that the trans encapsidation of a recombinant CVB3 virus was feasible using the cotransfection of a replicon plasmid, a plasmid expressing P1 capsid protein and a plasmid expressing T7 RNA polymerase. This produced titers of more than 10^6 gene transfer units/ml with no further concentration steps [14]. However, these recombinant viruses had a cytopathic effect on the cells. One of the main advantages of the viral vector system presented here is that the replication-defective rCVB3 vector does not induce any cytopathic effect on the cells subjected to gene delivery.

In our experiments, we have shown that the replication-defective rCVB3 vector can be generated for use as a novel gene transfer vector to express exogenous genes in cardiomyocytes without cytotoxicity. The novel rCVB3 vector system is potentially a highly useful tool for delivering genes to cardiomyocytes. Further optimization of the protocol and improvement of the viral stock titers are the objectives of upcoming studies.

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References


