J. Microbiol. Biotechnol. (2009), **19**(3), 307–313
doi: 10.4014/jmb.0803.237
First published online 13 August 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Systemic Analysis of a Novel Coxsackievirus Gene Delivery System in a Mouse Model

Kim, Yeon-Jung[1], Soo-Hyeon Yun[2], Byung-Kwan Lim[2], Ki-Bum Park[1], Ha-Na Na[1], Soo-Young Jeong[1], Dae-Sun Kim[1], Young-Joo Cho[1], Eun-Seok Jeon[2], and Jae-Hwan Nam[1]*

[1]*Department of Biotechnology, The Catholic University of Korea, Bucheon 420-743, Korea*
[2]*Department of Internal Medicine, Sungkyunkwan University, School of Medicine, Seoul 135-710, Korea*

Received: March 29, 2008 / Accepted: July 8, 2008

In order to systemically investigate the possibility of using coxsackievirus B3 (CVB3) to deliver foreign genes *in vivo*, a recombinant strain of CVB3 encoding the renilla gene (CVB3-renilla) was constructed. The recombinant CVB3 resulted in extensive and transient expression of the renilla protein within mouse organs, especially the pancreas. The level of expression was generally dependent upon the viral titer present. Moreover, the CVB3-renilla strain was completely attenuated. Interestingly, the recombinant CVB3 vector was expressed much more strongly in mouse organs than was a comparable adenoviral vector. The CVB3-renilla strain did not express the renilla gene in mice with pre-existing coxsackievirus-specific neutralizing antibodies, but direct organ-specific administration of the virus during open-peritoneum surgery was able to circumvent this immunity. This coxsackievirus vector may represent a useful means for delivering and expressing foreign genes in mouse models in an acute and extensive fashion.

Keywords: Coxsackievirus B3 (CVB3), gene delivery, viral vector

Coxsackievirus B3 (CVB3) is a picornavirus that contains a single-stranded positive-sense RNA genome that is approximately 7,400 nucleotides in length. The genome is translated into a long, single polypeptide chain that is then processed by a viral protease into four capsid proteins (VP4, VP2, VP3, and VP1), which are sequentially encoded at the genome's 5' end, and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) [11]. CVB3 induces either acute or chronic viral myocarditis in 5% to 50% of the children and young people infected, and its end-stage pathology can result in dilated cardiomyopathy [4, 10]. During viral infection of host cells, CVB3 interacts with the decay-accelerating factor (DAF), which acts as the virus's primary attachment coreceptor, and with the coxsackievirus and adenovirus receptor (CAR), which serves as the major internalization coreceptor. In fact, the tissue tropism of CVB3 may be related to the expression pattern of CAR in mouse organs [15].

Interestingly, previous studies have shown that CVB3 can act as a recombinant viral vector for the expression of exogenous genes encoding peptide [5, 6, 9, 17], cytokine [2, 7, 8], and GFP reporters [14]. Nevertheless, these studies have not systemically and quantitatively analyzed the characteristics of CVB3 vector gene expression, specifically the expression patterns within mouse organs and the duration of expression.

In this study, a recombinant coxsackievirus encoding a renilla reporter gene (CVB3-renilla) was constructed in order to quantitatively analyze its expression pattern in a mouse model. This recombinant coxsackievirus was able to transiently and extensively express the renilla protein in the heart, liver, spleen, and pancreas of the mouse hosts. In addition, the availability of CVB3-renilla expression in mouse organs was found to be greater than that of a recombinant adenovirus similarly encoded with renilla genes. To the best of our knowledge, this was the first study that systemically analyzed the efficiency of a recombinant coxsackievirus for the *in vivo* delivery of exogenous genes.

Materials and Methods

Cells and Viruses

A cervical cancer cell line, HeLa-UVM (hereinafter simply referred to as "HeLa" cells), was maintained in Dulbeccos modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum, 10,000 units/ml penicillin, and 10 mg/ml streptomycin. Cells were grown at 37°C in a humidified 5% CO_2 air atmosphere. The H3 Woodruff variant of the CVB3 virus was selected, and live virus was generated from the transfection of HeLa cells with infectious CVB3 cDNA [12]. After two freeze/thaw cycles, the virus was stored in aliquots at −85°C.

**Corresponding author*
Phone: +82-2-2164-4917; Fax: +82-2-2164-4865;
E-mail: jhnam@catholic.ac.kr

Recombinant Coxsackievirus cDNA

Modified cDNA encoding a recombinant CVB3 genome containing renilla genes was constructed as described previously [14]. The renilla gene was PCR amplified from the plasmid pRL-Null (Promega, Madison, WI, U.S.A.) with primers 5'-GCGTCGAATTCACTTCGAA-AGTTTATGATC-3' and 5'- GGTTCCTCGAGGGTTCATTTTTGAG-AACTCG-3'. These primers added EcoRI and XhoI cut sites upstream and downstream of the gene, respectively. To construct the recombinant coxsackievirus encoding the renilla gene, we used the previously published CVB3-H3 variant cDNA in pBluescript [12], and used the cDNA sequence for the renilla gene of the pRL-Null. The CVB3 genome was modified to include an in-frame synthetic polylinker containing EcoRI and XhoI sites in order to insert the renilla cDNA.

Viral Titration

The recombinant CVB3 was propagated in HeLa cells, and the viral titer was quantified using previously reported standard plaque assay methods for HeLa cells [12].

Immunostaining

Tissue samples were fixed with 4% formaldehyde and embedded in paraffin. The samples were sectioned into 5-μm-thick slices and stained with hematoxylin-eosin (H&E) in accordance with the protocol outlined in a previous study [12].

ELISA for Cytokine Detection

Serum cytokine levels were determined using commercial ELISA kits (IL-4: eBioscience, Dan Diego, CA, U.S.A.; IL-6: BD Bioscience, Franklin Lakes, NJ, U.S.A.) in accordance with the manufacturer's protocol.

Detection of Renilla Gene Expression

Tissues were homogenized in DMEM containing 1% antibiotics, using a Dounce homogenizer. Cellular debris was removed *via* centrifugation at 10,000 ×*g* for 10 min at 4°C, and renilla expression was assayed using the resulting supernatant in accordance with the manufacturer's protocol (Promega).

RESULTS

Construction of Recombinant Coxsackievirus Encoding Renilla Genes

An infectious cDNA clone of coxsackievirus B3 was employed to construct a recombinant coxsackievirus encoding the renilla gene. The renilla gene was inserted into the cDNA genome between the 5' untranslated region (UTR) and the VP4 coding sequence. All cloning procedures and artificial amino acid positions were identical to those employed in

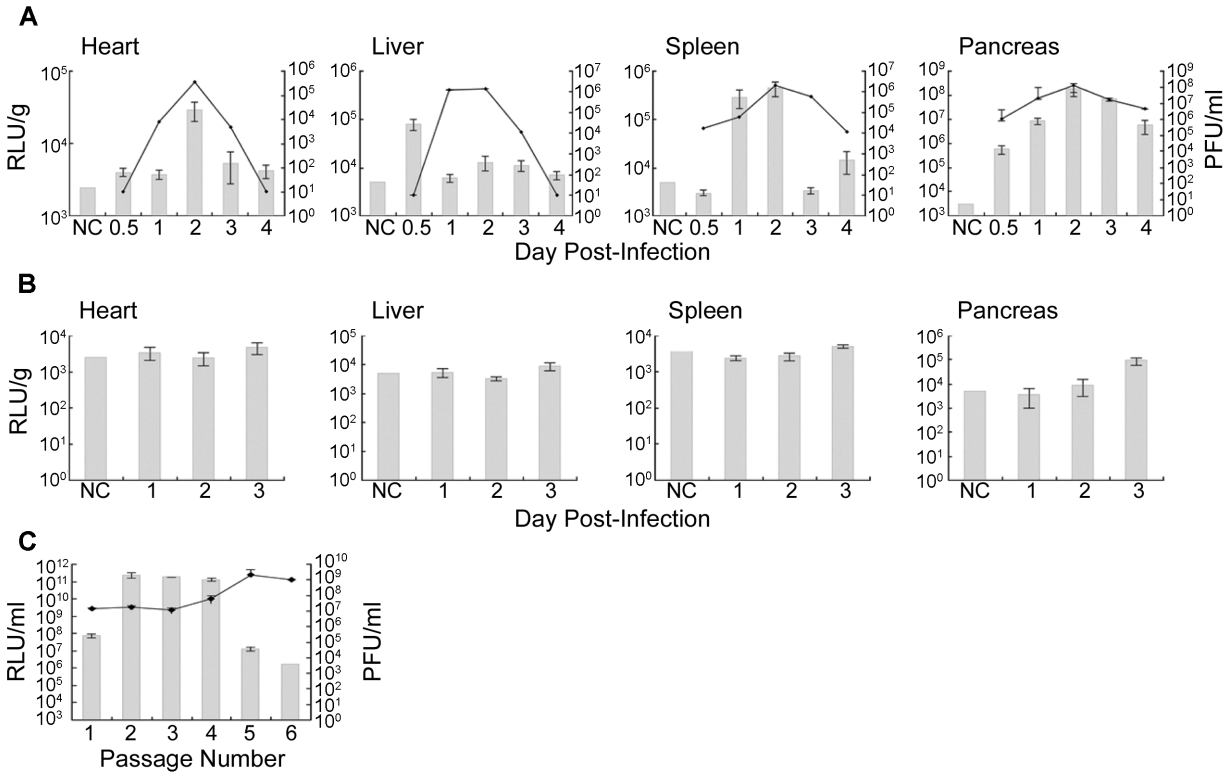

Fig. 1. Expression pattern of recombinant coxsackievirus encoding the renilla gene (CVB3-renilla) in mouse organs.
A. Renilla gene expression in mice given an intraperitoneal injection of 1×10^7 PFU. **B**. Renilla gene expression in mice given a subcutaneous injection of 1×10^7 PFU. **C**. Stability of renilla expression by CVB3-renilla-infected HeLa cells. HeLa cells were exposed to CVB3 at an moi of 1, and at 24 h post-infection the supernatant of the infected cells was used to infect new HeLa cells. This cycle of infection was repeated in a serial fashion. The viral titer of each supernatant and the renilla expression of each cell lysate were measured. NC indicates the negative control group of non-infected mice given a mock injection. Bars indicate renilla expression (RLU; left Y-axis) and lines indicate viral titer (PFU; right Y-axis)

previous studies [14]. The titer of recombinant coxsackievirus (CVB3-renilla) reached 5×10^8 plaque-forming units (PFU)/ml in HeLa cells, and this was 4-fold lower than that of the wild-type CVB3 H3 variant (2×10^9 PFU/ml). The virus stock was stored at −85°C for use in further studies.

Expression Pattern of Renilla Protein in Mice Injected with CVB3-Renilla

The recombinant CVB3-renilla virus was administered to 5 mice within each experimental group *via* intraperitoneal (IP) injection at a dosage of 1×10^7 PFU/mouse. The mouse organs were harvested at the indicated times post-infection, and the tissues' renilla expression levels were measured. Renilla expression was transient and acute (Fig. 1A). Expression was approximately 12-, 16-, 100-, and 70,000-folds higher in the heart, liver, spleen, and pancreas, respectively, than in the same tissues from control group mice, which had not been injected with the recombinant virus. Pancreatic tissues demonstrated a particularly prolonged period of expression, and this may have been related to the fact that viral titers were highest in this organ. Subcutaneous (SC) injections with 1×10^7 PFU CVB3-renilla/mouse resulted in low renilla expression in mouse organs (Fig. 1B). In addition, very low viral titers were detected in these mice's organs (data not shown). This indicated that the means of virus administration was of great importance in determining the levels of the exogenous gene expression using a recombinant coxsackievirus vector.

As previously mentioned, renilla expression from CVB3-renilla infection was generally dependent upon the viral titer found in specific organs. In the liver and spleen tissues, however, the levels of renilla expression did not perfectly correlate with the viral titers found in these organs (Fig. 1A). We speculated that the recombinant coxsackievirus' ability to express foreign genes might in fact be dependent on viral replication. To test this hypothesis, we infected HeLa cells with CVB3-renilla at a multiplicity of infection (moi) of 1. The supernatant from the infected cells was then injected into new HeLa cells to start a new round of infection, and this process was repeated in a serial fashion. The level of renilla expression decreased dramatically after five passages even though high viral titers were still found in the supernatants. This suggested that the genetic stability of renilla expression was maintained only through five rounds of infection.

Inhibitory Effect of Pre-Existing Coxsackievirus Antibodies on Renilla Expression and Restoration of Susceptibility Through Local Organ-Specific Administration

Because the coxsackievirus is prevalent in human populations, coxsackievirus-specific antibodies are commonplace in human subjects. This raised concerns as to whether these pre-existing CVB3 antibodies may prevent recombinant coxsackievirus infection and the subsequent expression of exogenous genes. To examine this possibility, we immunized mice with the highly attenuated mutant coxsackievirus strain YYFF with the two phenylalanine residues found in the VP2 (Y240 and Y254) (unpublished data by Kim *et al.*) at a dosage of 1×10^6 PFU/mouse. After 14 days, a dose 1×10^7 PFU of CVB3-renilla was administered to each mouse *via* IP injection. The renilla expression was then measured at 1 and 2 days post-infection. At 14 days post-immunization with YYFF, the titer of the neutralizing antibodies reached

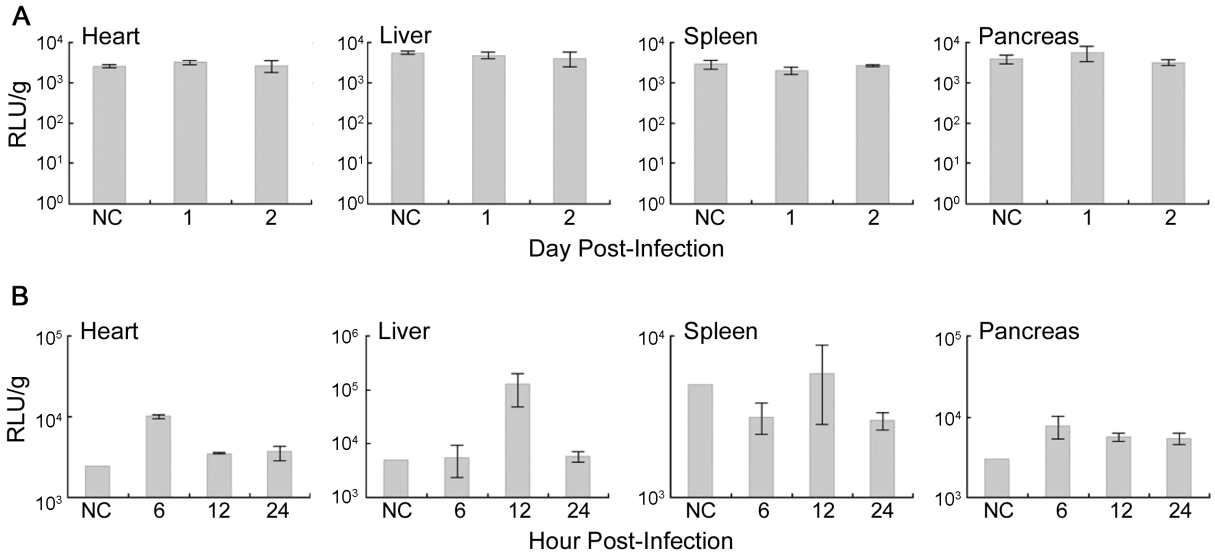

Fig. 2. Inhibitory effects of pre-existing coxsackievirus-specific antibodies on renilla expression.
A. CVB3-renilla exposure did not result in renilla gene expression in mice that had been pre-immunized with YYFF. **B**. Renilla expression was achieved in mice with pre-existing CVB3-specific antibodies by means of a direct injection into the subject's liver during open-peritoneum surgery. NC indicates the negative control group of non-infected mice given a mock injection.

1:160 (50% reduction). Renilla expression was completely curtailed within the organs of mice infected with CVB3-renilla *via* IP injection. However, renilla expression in liver tissue was achieved when a 1×10^7 PFU dose of CVB3-renilla was directly injected into the liver of each mouse using a method of local administration that involved open-peritoneum surgery. Expression increased approximately 25-fold over the case of mock injection (NC; negative control) (Fig. 2B).

Comparison of *In Vivo* Expression Patterns of Adenovirus Encoding Renilla Genes (Ad-Renilla) to Those of CVB3-Renilla

As coxsackievirus is an RNA virus, its genome remains extrachromosomal, thus minimizing the risk of insertional mutagenesis [18]. This means that even though a recombinant retrovirus is capable of continuously expressing foreign genes, the recombinant coxsackievirus may not be able to do so [19]. Because of its high replication titers, its efficiency of infection, and its low levels of vector genome integration, adenovirus vectors are currently the most widely used viral vector system [3, 13, 16]. Therefore, we compared the *in vivo* expression patterns of CVB3-renilla with those of an adenovirus vector encoding the renilla gene (Ad-renilla), which was purchased from Newgex Inc. Exposure of HeLa cells to an Ad-renilla moi of 1 resulted in the efficient *in vitro* expression of the renilla gene at 24 h post-infection (Fig. 3A). The levels of expression from Ad-renilla and CVB3-renilla infection were found to be similar, and both samples reached 1×10^8 RLU/ml at 24 h post-infection (Figs. 3A and 1C).

Intravenous (IV) injection was superior to IP injection for purposes of *in vivo* exogenous gene expression using Ad-renilla (data not shown). We compared the expression patterns of both Ad-renilla and CVB3-renilla following IV injection in the mouse model. Both recombinant viruses were injected at a dosage of 1×10^7 PFU/mouse. In the heart and liver, CVB3-renilla showed similar levels of expression to Ad-renilla (Figs. 3B and 3C). In the pancreas, however, CVB3-renilla expression was approximately 10- to 1,000-

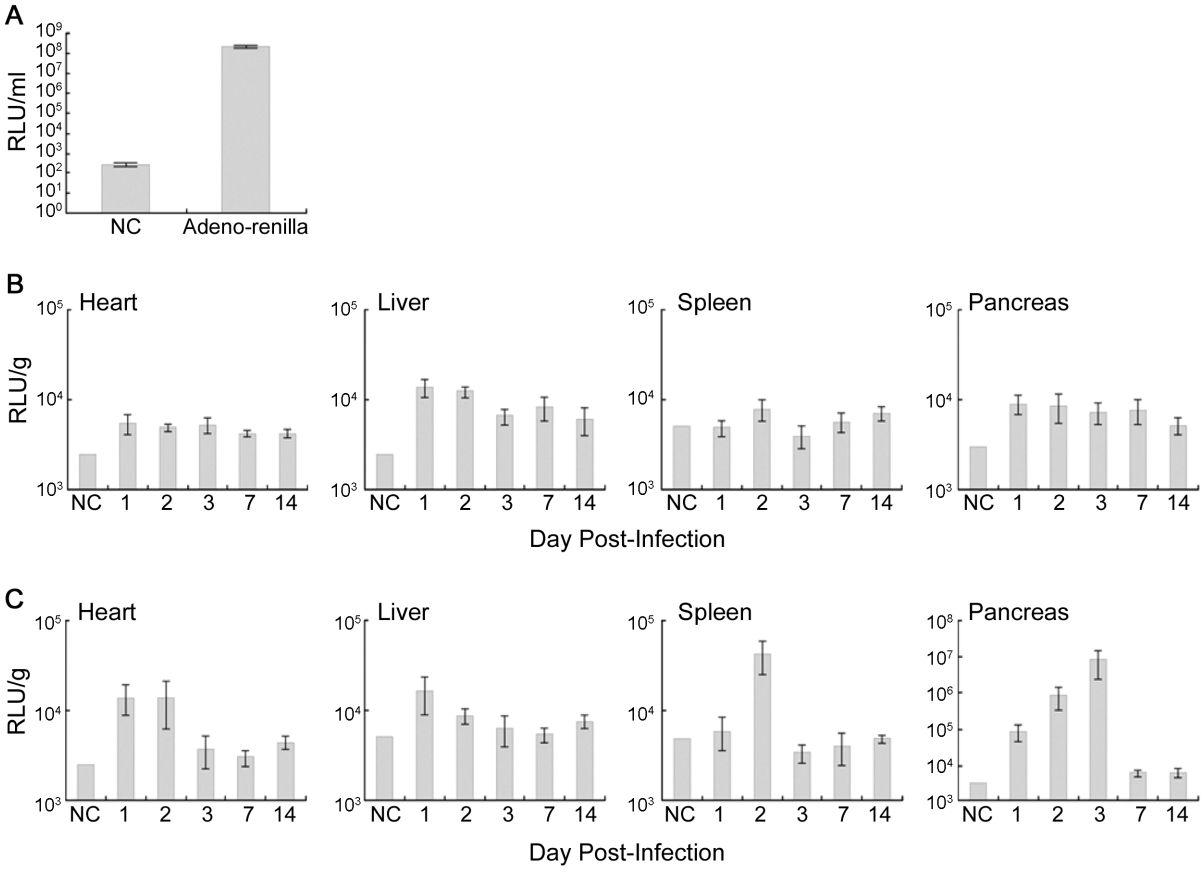

Fig. 3. Comparison of renilla expression in different mouse organs with CVB3 renilla and a recombinant adenovirus encoding the renilla gene (Ad-renilla).

A. Renilla gene expression in HeLa at 24 h post-infection with Ad-renilla. **B**. Renilla gene expression in mice given an IV injection of 1×10^7 PFU of Ad-renilla. **C**. Renilla gene expression in mice given an IV injection of 1×10^7 PFU of CVB3-renilla. NC indicates the negative control group of non-infected mice given a mock injection.

folds higher than Ad-renilla expression after the same amount of time post-infection.

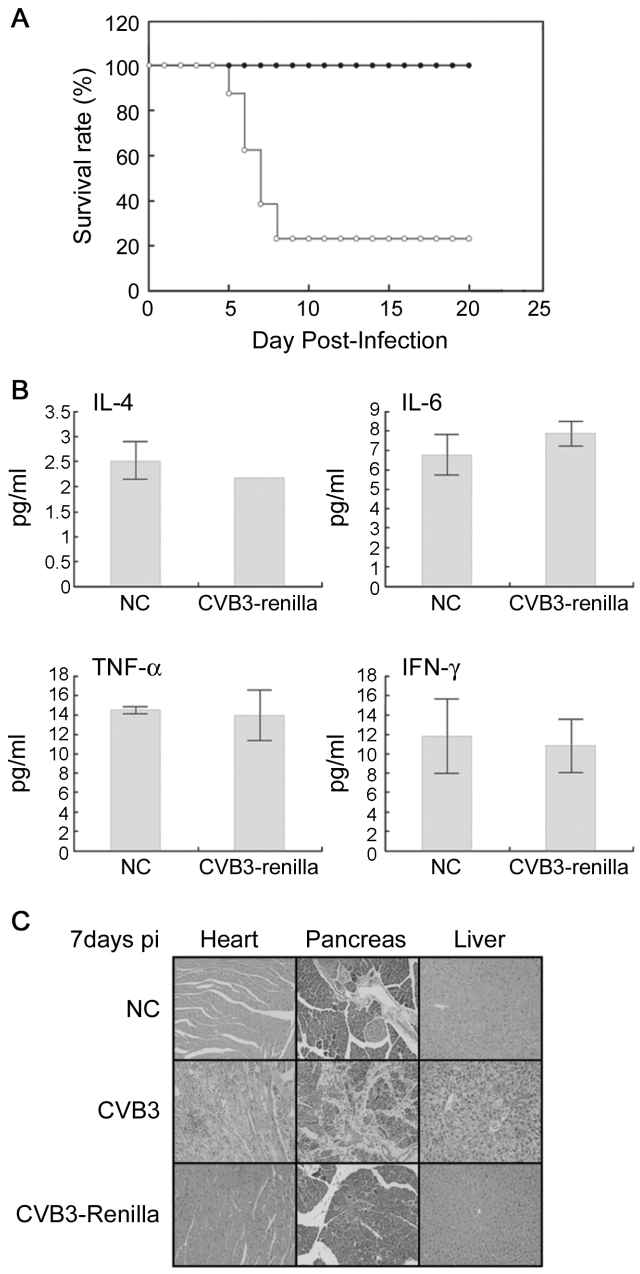

Fig. 4. Attenuation of CVB3-renilla *in vivo*.
A. Survival rate of mice infected with 1×10^7 PFU/mouse of CVB3-renilla and wild-type CVB3. Each group consisted of 10 mice. Open circles correspond to the wild-type CVB3, and closed circles correspond to the CVB3-renilla. **B**. The induction of inflammatory cytokines in the serum of mice infected with CVB3-renilla. **C**. Histology of inflammation in mice infected with CVB3-renilla and wild-type CVB3. Staining was performed using hematoxylin-eosin (H&E) in order to measure inflammation patterns in the heart, pancreas, and liver. The blue-purple regions indicate a basophilic structure of infiltrated cells, and the bright pink areas represent the eosinophilic cytoplasm of myocardial cells. Whereas CVB3 exhibited vastly inflamed areas and massive destruction of exocrine pancreatic tissues, CVB3-renilla did not. All data in **B** and **C** were taken from mice at 7 days post-infection.

Attenuation of the Recombinant Coxsackievirus

It was previously reported that recombinant coxsackieviruses encoding foreign genes usually become attenuated [8, 14]. We examined the extent of viral attenuation in the CVB3-renilla recombinant strain. Mice infected with the virus maintained a 100% survival rate (Fig. 4A). Infection was asymptomatic; the usual symptoms of wild-type CVB3 infection, such as reduction in body weight and loss of fur, were not observed (data not shown). In addtion, CVB3-renilla infection did not induce the release of inflammatory cytokines IL-4, IL6, TNF-α, and IFN-γ into the host's serum (Fig. 4B). Whereas H&E staining of the heart, pancreas, and liver tissue of mice infected with CVB3-renilla did not show any of the typical signs of inflammation, the tissues of those mice infected with wild-type CVB3 did show signs of serious inflammation at day 7 post-infection (Fig. 4C). It was therefore concluded that the CVB3-renilla strain was clearly attenuated *in vivo*.

DISCUSSION

Previous studies have demonstrated that CVB3 vectors may be a useful means for delivering foreign genes to mouse cells *in vivo* [7, 8]. Henke *et al*. [8] showed that recombinant CVB3 could deliver genes encoding IL-10 and IFN-γ to the pancreas and heart. Chapman *et al.* [2] found that CVB3 could deliver the IL-4 gene to mouse heart, pancreas, and liver tissue. Both studies were able to establish the presence of the recombinant viral genome and the translation of reporter proteins corresponding to the foreign genes within the infected mouse tissues using RT-PCR and immunohistochemistry, respectively. These studies did not, however, measure the protein expression levels or the duration of exogenous gene expression within the infected mouse organs. In this study, expression of the renilla reporter gene was quantitatively measured using a commercial renilla detection system. Whereas the renilla gene was expressed acutely and transiently for just one or two days within the heart, liver, and spleen, it was continuously expressed within the pancreas for more than 4 days post-injection and reached the highest expression level among all tissues examined, 10^6 to 10^8 RLU/g (Fig 1A). In experiments that somewhat contradicted these results, Lim *et al.* [14] detected GFP reporter protein expressed by a recombinant coxsackievirus vector in heart tissue at 8 weeks post-injection. This conflict may be explained by the nature of the expressed reporter proteins and the detection system employed to quantify them. Lim *et al.* [14] used immunohistochemistry to detect GFP in heart tissue, and because the half-life of intracellular GFP is rather long, it is possible the GFP detected after 8 weeks post-injection was residual rather than newly expressed protein. This justification is of course based on the assumption

that the heart is a post-mitotic organ incapable of regenerating its parenchyma cells [1]. Furthermore, this particular aspect of their data was not related to their findings regarding the virus titer, and the virus had already disappeared from the heart by 8 weeks post-injection. In this study, we used a chemical detection system that depends on reporter protein function to measure renilla gene expression. In addition, the level of renilla gene expression generally correlated with viral titer measurements. Furthermore, levels of expression in heart tissue that were about 2-fold higher than those found in negative controls were maintained after the signal peak at 2 days post-injection (Fig. 1A). This implied that, at the very least, the CVB3 vector can transiently deliver the target gene to mouse organs, especially the pancreas. In terms of the injection route, IP was found to be an effective method for administering the virus to mice (Figs. 1A and 1B). However, because IP injection is not an adequate method of administration for human subjects, IV injection should be much more reliable (Fig. 3C).

There are many important safety concerns regarding the use of recombinant coxsackieviral vectors in human trials. Infected mice maintained a 100% survival rate, and infection did not result in any of the symptoms typically associated with the wild-type pathology, such as reduction in body weight and loss of fur (data not shown). Furthermore, the recombinant coxsackievirus constructed in this study did not induce inflammatory cytokine production, and infection did not result in any typical signs of inflammation in the affected mouse tissues (Fig. 4). There results agreed with previous studies, which found that other recombinant coxsackieviruses encoding foreign genes were also attenuated [8, 14]. The mechanism behind this attenuation effect is unknown. We hypothesized that, unlike wild-type viral variant H3, the encoding of foreign genes may slow viral replication, and the resulting lower viral titers, which were indeed observed in mouse organs (data not shown), were responsible for the attenuation. We were also able to circumvent pre-existing CVB3-specific host antibodies that can interfere with expression, by performing a direct injection, which involved local organ administration of the virus during open-peritoneum surgery (Fig. 2). This implied that this viral vector system could be applied to specific organs in this manner, even if some CVB3-neutralizing antibodies were circulating *in vivo*.

Ultimately, the most interesting finding of this study was the fact that CVB gene delivery was more efficient than adenoviral delivery, especially in pancreatic tissues (Fig. 3). Although adenovirus has been the most commonly used vector for *in vivo* gene delivery, it still exhibits obvious limitations in terms of efficient transduction and the duration of gene expression [3, 13]. Although dosages of adenovirus in excess of 10^{10} PFU/mouse are typically used to efficiently deliver foreign genes to mice, this study employed a lower viral dosage of 10^7 PFU/mouse to compare adenovirus and coxsackievirus vectors. This comparison proved that the coxsackievirus vector was more efficient in delivering genes to mouse organs, and this advantage was particularly evident in pancreatic tissues (Fig. 3).

In conclusion, recombinant CVB3 encoding foreign genes may represent an efficient system for gene delivery *in vivo*, especially to the pancreas. In addition, the recombinant coxsackievirus system has proven to be more effective than the corresponding adenovirus system at lower viral titers.

Acknowledgments

This work was supported by a grant from the Gyeonggi Regional Research Center (GRRC) of the Catholic University of Korea, the Korea National Institutes of Health, Korea Food & Drug Administration, and by the MKE & KOTEF through the Human Resource Training Project for Strategic and Technology.

References

1. Anversa, P., J. Kajstura, A. Leri, and R. Bolli. 2006. Life and death of cardiac stem cells: A paradigm shift in cardiac biology. *Circulation* **113:** 1451–1463.
2. Chapman, N. M., K. S. Kim, S. Tracy, J. Jackson, K. Höfling, J. S. Leser, J. Malone, and P. Kolbeck. 2000. Coxsackievirus expression of the murine secretory protein interleukin-4 induces increased synthesis of immunoglobulin G1 in mice. *J. Virol.* **74:** 7952–7962.
3. Douglss, J. T. 2007. Adenoviral vectors for gene therapy. *Mol. Biotechnol.* **36:** 71–80.
4. Esfandiarei, M. and B. M. McManus. 2007. Molecular biology and pathogenesis of viral myocarditis. *Annu. Rev. Pathol.* **3:** 127–155.
5. Halim, S. S., D. N. Collins, and A. I. Ramsingh. 2000. A therapeutic HIV vaccine using coxsackie-HIV recombinants: A possible new strategy. *AIDS Res. Hum. Retroviruses* **16:** 1551–1558.
6. Halim, S. S., S. E. Ostrowski, W. T. Lee, and A. I. Ramsingh. 2000. Immunogenicity of a foreign peptide expressed within a capsid protein of an attenuated coxsackievirus. *Vaccine* **19:** 958–965.
7. Henke, A., N. Jarasch, U. Martin, J. Wegert, A. Wildner, R. Zell, and P. Wutzler. 2008. Recombinant coxsackievirus vectors for prevention and therapy of virus-induced heart disease. *Int. J. Med. Microbiol.* **298:** 127–134.
8. Henke, A., R. Zell, G. Ehrlich, and A. Stelzner. 2001. Expression of immunoregulatory cytokines by recombinant coxsackievirus B3 variants confers protection against virus-caused myocarditis. *J. Virol.* **75:** 8187–8194.
9. Höfling, K., S. Tracy, N. Chapman, K. S. Kim, and J. Smith Leser. 2000. Expression of an antigenic adenovirus epitope in a group B coxsackievirus. *J. Virol.* **74:** 4570–4578.
10. Kawai, C. 1999. From myocarditis to cardiomyopathy: Mechanisms of inflammation and cell death: Learning from the past for the future. *Circulation* **99:** 1091–1100.
11. Kim, K. S., G. Hufnagel, N. M. Chapman, and S. Tracy. 2001. The group B coxsackieviruses and myocarditis. *Rev. Med. Virol.* **11:** 355–368.

12. Knowlton, K. U., E. S. Jeon, N. Berkley, R. Wessely, and S. Huber. 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. *J. Virol.* **70:** 7811–7818.
13. Kreppel, F. and S. Kochanek. 2008. Modification of adenovirus gene transfer vectors with synthetic polymers: A scientific review and technical guide. *Mol. Ther.* **16:** 16–29.
14. Lim, B. K., J. O. Shin, S. C. Lee, D. K. Kim, D. J. Choi, S. C. Choe, K. U. Knowlton, and E. S. Jeon. 2005. Long-term cardiac gene expression using a coxsackieviral vector. *J. Mol. Cell. Cardiol.* **38:** 745–751.
15. Maisch, B., A. D. Ristiæ, G. Hufnagel, and S. Pankuweit. 2002. Pathophysiology of viral myocarditis: The role of humoral immune response. *Cardiovasc. Pathol.* **11:** 112–122.
16. Mukai, E., S. Fujimoto, F. Sakurai, K. Kawabata, M. Yamashita, N. Inagaki, and H. Mizuguchi. 2007. Efficient gene transfer into murine pancreatic islets using adenovirus vectors. *J. Control. Release* **119:** 136–141.
17. Slifka, M. K., R. Pagarigan, I. Mena, R. Feuer, and J. L. Whitton. 2001. Using recombinant coxsackievirus B3 to evaluate the induction and protective efficacy of CD8+ T cells during picornavirus infection. *J. Virol.* **75:** 2377–2387.
18. Tam, P. E. 2006. Coxsackievirus myocarditis: Interplay between virus and host in the pathogenesis of heart disease. *Viral Immunol.* **19:** 133–146.
19. Van den Haute, C., K. Eggermont, B. Nuttin, Z. Debyser, and V. Baekelandt. 2003. Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain. *Hum. Gene Ther.* **14:** 1799–1807.