Prolonged gene expression in mouse lung endothelial cells following transfection with Epstein–Barr virus-based episomal plasmid

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The development of a strategy to deliver a gene to pulmonary endothelium will be useful for gene function study and for pulmonary gene therapy. Cationic lipidic vectors are efficient in gene transfer to pulmonary endothelium via the vascular route; however, gene expression is transient and lasts for only a few days. In this study, we show that pulmonary gene transfer via cationic lipidic vectors can be significantly improved using an Epstein–Barr virus (EBV)-based expression plasmid. Systemic administration of cationic liposomes followed by the EBV-based plasmid led to gene expression in the lung that lasted for more than 3 weeks. Prolonged and high levels of gene expression can also be obtained in primary mouse lung endothelial cells (MLEC) following lipofection with an EBV-based plasmid. These results suggest the utility of this gene transfer protocol in studying the expression of cloned genes in lung endothelial cells and in pulmonary gene therapy.

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Somatic gene transfer to the pulmonary endothelium may be a useful strategy for modifying the phenotype of endothelium and/or vascular smooth muscle in disorders such as primary pulmonary hypertension, ARDS, or pulmonary metastatic disease. It may also provide a useful research tool to study the function of cloned genes in pulmonary endothelial cells in vitro and in vivo. Among the viral vectors employed for such potential use,1,2 adenoviral vectors have proved to be limited with respect to efficiency in part because of difficulty in assuring significant residence time in the lung and/or paucity of receptors for adenovirus on endothelium. Also, adenoviral vectors are only moderately effective in infecting primary mouse lung endothelial cells in vitro. First-generation cationic lipidic vectors also produce modest degrees of gene transfer to lung after i.v. administration.3,4 Modification of cationic lipid composition and DNA/lipid ratios led to considerably higher gene transfer efficiency in the lung with the endothelial cells being the major cell type transfected.5–10 However, gene expression in the lung via this gene transfer protocol is transient and lasts for only a few days. In the current study, we investigate whether in vitro and in vivo gene transfer protocol to mouse lung endothelial cells (MLEC) can be significantly improved using an EBV-based expression plasmid.

EBV plasmid is a replicating episomal vector that has been developed to overcome the problem of rapid elimination of intracellularly delivered plasmid DNA in nonviral vector-mediated gene transfer. The viral elements required for episomal replication and nuclear retention are the cis-acting replication origin (oriP) of the EBV gene and the EBV nuclear antigen-1 (EBNA-1), which interacts with the oriP region.11–13 Plasmids containing the oriP and EBNA-1 sequences are maintained as low-copy number DNA episomes in the cell nucleus and replicate once per cell cycle in primate cells. Most studies suggest that long-term replication of EBV-derived plasmids occurs only in primate cells but not in rodent cells.11,14 Nonetheless, a more persistent gene expression was found in a number of rodent tissues following nonviral method-mediated delivery of EBV plasmid compared to non-EBV plasmid possibly because of the nuclear retention function and the enhancer activity of transgene expression induced by EBNA-1.15–18 We hypothesize that lipofection of MLEC can also be significantly improved both in vitro and in vivo using the EBV-based plasmid.

Figure 1 shows the maps of three different plasmids used in this study. Plasmids pGEG.GL3 and pG.GL3 contain the firefly luciferase gene under the control of a CAG promoter. pGEG.GL3 also contains an EBV EBNA-1 gene driven by a CAG promoter. In addition, pGEG.GL3 contains the EBV oriP. Plasmid pNGVL3-Luc contains the firefly luciferase gene under the control of a cytomegalovirus (CMV) promoter. In the initial experiment, we compared the level of gene expression in the lung with
pGEG.GL3 using two different gene transfer protocols, that is, complex injection and sequential injection. With a non-EBV plasmid, we have shown recently that sequential injection of cationic liposomes and plasmid is more efficient in pulmonary gene transfer than injection of cationic liposome/DNA complexes. Furthermore, sequential injection is associated with a significantly reduced proinflammatory cytokine response. A similar result was observed in this study with an EBV-based plasmid. As shown in Figure 2a, serum levels of TNF-α in sequential injection group were only 30% of those in complex injection group. Furthermore, sequential injection of DOTAP:cholesterol liposomes and pGEG.GL3 led to a significantly higher level of gene expression in the lung than complex injection (Figure 2b). We and others have shown that proinflammatory cytokines can significantly inhibit transgene expression in either viral or nonviral vector-mediated gene transfer. The improved pulmonary gene transfer via sequential injection protocol is probably because of a decreased inhibitory effect of cytokines on transgene expression. Thus, sequential injection protocol was used in subsequent studies.

Figure 3 shows the luciferase expression in the lungs over time following sequential injection of DOTAP:cholesterol liposomes (+/− charge ratio of 6:1) followed by

**Figure 1** Plasmid structure: (a) pG.GL3, (b) pGEG.GL3, (c) pNGVL3-Luc. CAG: the chicken β-actin promoter-cytomegalovirus (CMV) enhancer; EBNA-1: Epstein-Barr nuclear antigen-1; oriP: EBV latent origin of replication; Luc: luciferase.

**Figure 2** TNF-α cytokine response (a) and luciferase gene expression in mouse lungs (b) following i.v. injection of cationic liposome/DNA complexes or cationic liposomes followed by plasmid DNA. DOTAP:cholesterol liposome/pGEG.GL3 complexes were prepared at a +/− charge ratio of 6/1 as described and injected into female CD-1 mice (Charles River Laboratories, Wilmington, MA, USA; eight mice in each group) via tail vein at a dose of 35 μg DNA per mouse. In a different group, mice received tail vein injection of DOTAP:cholesterol liposomes (1.3 mmol lipid/mouse) followed by pGEG.GL3 (35 μg/mouse). At 2 h following injection, mice were bled from the retro-orbital sinuses under anesthesia. The blood was allowed to stay at 4°C for 4 h and then centrifuged at 14,000 g for 10 min at 4°C. Serum levels of TNF-α were determined with the specific cytokine immunoassay kit (R&D Systems, Minneapolis, MN, USA). In a separate experiment, groups of eight mice received i.v. injection of DOTAP: cholesterol liposome/pGEG.GL3 complexes or sequential injection of DOTAP:cholesterol liposome followed by pGEG.GL3 as described above. At days 1 and 3 following injection, mice were killed and lungs were removed and homogenized in 1 ml of ice-cold lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris, pH 7.8) with a tissue tearer for 20 s at high speed. The homogenates were then centrifuged at 14,000 g for 10 min at 4°C. Of the supernatant 10 μl was analyzed with the luciferase assay system (Promega, Madison, WI, USA) using an automated LB953 luminometer (Berthold, Bad Wildbad, Germany). The protein content of the supernatant was measured with the two-tailed unpaired Student’s t-test using the PRISM software program (GraphPad Software, San Diego, CA, USA). *P<0.05; **P<0.01 (versus complex injection).
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Figure 3 Prolonged gene expression in mouse lungs following sequential injection of cationic liposomes and an EBV-based plasmid. Groups of eight mice received tail vein injection of DOTAP:cholesterol liposomes (1.3 mmol lipid/mouse) followed by pGEG.GL3, pG.GL3, or pNGVL3-Luc (35 µg/mouse). At different time points, mice were killed and the level of gene expression in the lung was assayed as described in the legend to Figure 2. The level of luciferase activity in lungs of mock-transfected mice is less than 1000 RLU/mg protein. *P<0.05 (versus pG.GL3).

pGEG.GL3, pG.GL3, or pNGVL3-Luc (35 µg/mouse). Peak expression of luciferase occurred on day 1 for all plasmids. However, of the three plasmids, gene expression in pNGVL3-Luc group declined most rapidly with time and was not different from background by 21 days. Gene expression in the pGEG.GL3 group declined during the first week following sequential injection, but then there was a rebound in gene expression over the subsequent 2 weeks. A similar phenomenon was observed in a study with complex injection. The rapid decline in gene expression in the first few days following injection is probably largely because of the inhibitory effect of cytokines. Despite a decreased cytokine response in sequential injection (Figure 2a), the level of TNF-α in the serum might be sufficient to cause significant inhibition on transgene expression. Nevertheless, the levels of gene expression in pGEG.GL3 group were significantly higher than those in pG.GL3 or pNGVL3-Luc group at all time points examined. Of note is that even after 3 weeks, there is a detectable level of gene expression in lungs of mice injected with pGEG.GL3.

Having characterized pulmonary gene transfer with pGEG.GL3, the sequential injection protocol was further evaluated using pGEG.EGFP as a reporter gene. No green cells were observed in the lungs of mice treated with a control plasmid (pGEG.GL3) (data not shown). In contrast, there was localized gene expression throughout the distal lung of mice that received pGEG.EGFP (Figure 4a). The cell type of transfected cells was further analyzed by anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) immunofluorescence staining of the lung sections. Constitutive expression of PECAM-1 is a fundamental characteristic of endothelial cells. As shown in Figure 4a and b, EGFP signal was substantially colocalized with PECAM labeling, confirming that endothelial cells were the major cell type transfected.

We then examined whether primary MLEC can also be efficiently transfected via EBV-based plasmid. Primary MLEC were prepared by modification of an immuno-bead protocol. Briefly, mouse lungs were finely minced and digested in collagenase (Type I, 100 µg/ml). Cell suspensions were incubated with a monoclonal antibody (rat anti-mouse) to PECAM-1 (BD Pharmingen, San Diego, CA, USA) for 30 min at 4°C. The cells were washed twice with buffer to remove unbound antibody, and resuspended in binding buffer containing the appropriate number of washed magnetic beads coated with sheep anti-rat IgG (Jackson ImmunoResearch Laboratories) for 1 h at RT. The sections were mounted in Gelvatol (Monsanto, St Louis) and the images were collected using a Leica TCS NT confocal microscope at 1024 x 1024 pixel resolution.

One of the potential concerns over the use of EBV plasmid is the oncogenicity of EBNA-1 protein. Transgenic mice harboring the EBNA-1 gene driven by B lymphocyte-specific enhancer developed B-cell lymphoma. However, several studies have shown that EBNA-1...
homogenate centrifuged at 14,000 g for 10 min at 4°C and the supernatant
and 11 following transfection, the cells were lysed using ice-cold lysis
buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris, pH 7.8), the
homogenate centrifuged at 14,000 g for 10 min at 4°C and the supernatant
analyzed for luciferase expression. *P < 0.05 (versus pG.GL3).

Figure 5 Prolonged gene expression in primary lung endothelial cells
following lipofection with an EBV-based plasmid. Primary MLEC were
prepared as described in the text. Cells of density 1 × 10^6 cells/well in a 48-
well plate were transfected with 1.5 μg of plasmid (pGEG.GL3, pG.GL3, or
pNGVL3-Luc) complexed to DOTAP:cholesterol liposomes at a +/−
charge ratio of 2:1 in serum-free medium. At 4 h later, the transfection
medium was removed and replaced with complete medium. At days 1, 4, 7,
and 11 following transfection, the cells were lysed using ice-cold lysis
buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris, pH 7.8), the
homogenate centrifuged at 14,000 g for 10 min at 4°C and the supernatant
analyzed for luciferase expression. *P < 0.05 (versus pG.GL3).

itself is insufficient for B lymphocyte transformation in vitro in the absence of the latent viral properties EBNA-
2,29 EBNA-3A,30 EBNA-3C,30 and LMP-31. Furthermore, 293 cells stably transfected with EBNA-1 did not grow in
soft-agar plates and were sensitive to serum depletion.32

It is unlikely that the current gene expression system will
induce tumor in the host. Further studies are required to
address the safety of long-term gene therapy using EBV
plasmid.

In summary, we have shown that systemic adminis-
tration of cationic liposomes followed by an EBV
plasmid led to a prolonged gene expression in the lung,
which lasted for more than 3 weeks. An efficient
transfection of primary MLEC can also be achieved
using EBV plasmid. These results suggest the utility of
this gene transfer protocol in studying the expression of
cloned genes in lung endothelial cells and in pulmonary
gene therapy.

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References
1 Lemarchand P et al. In vivo adenosine-virus-mediated gene transfer
to lungs via pulmonary artery. J Appl Physiol 1994; 76: 2840–
2845.
2 Rodman DM et al. In vivo gene delivery to the pulmonary
circulation in rats: transgene distribution and vascular inflam-
matory response. Am J Respir Cell Mol Biol 1997; 16: 640–649.
3 Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after
intravenous DNA delivery into adult mice. Science 1993; 261:
209–211.
4 Canonico AE, Conary JT, Meyrick BO, Brigham KL. Aerosol and
intravenous transfection of human alpha 1-antitrypsin gene to
lungs of rabbits. Am J Respir Cell Mol Biol 1994; 10: 24–29.
5 Liu Y et al. Factors influencing the efficiency of cationic
liposome-mediated intravenous gene delivery. Nat Biotechnol
1997; 15: 167–173.
6 Liu F, Qi H, Huang L, Liu D. Factors controlling efficiency of
cationic lipid-mediated transfection in vivo via intravenous
administration. Gene Ther 1997; 4: 517–523.
7 Templeton NS et al. Improved DNA:liposome complexes for
increased systemic delivery and gene expression. Nat Biotechnol
1997; 15: 647–652.
8 Li S, Huang L. In vivo gene transfer via intravenous adminis-
tration of cationic lipid–protamine–DNA (LPD) complexes. Gene
Ther 1997; 4: 891–900.
9 Barron LG, Meyer KB, Szoka Jr FC. Effects of complement
depletion on the pharmacokinetics and gene delivery mediated
by cationic lipid–DNA complexes. Hum Gene Ther 1998; 9:
315–323.
10 Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of
cationic liposome–plasmid DNA complexes by polyamines and
poly(ethylene glycol)-phospholipid conjugates for efficient in
vivo gene delivery. FEBs Lett 1997; 400: 233–237.
11 Yates JL, Warren N, Sugden B. Stable replication of plasmids
derived from Epstein–Barr virus in various mammalian cells.
Nature 1985; 313: 812–813.
12 Lupton S, Levine AJ. Mapping genetic elements of Epstein–Barr
virus that facilitate extrachromosomal persistence of Epstein–
Barr virus–derived plasmids in human cells. Mol Cell Biol 1985; 5:
2533–2542.
13 Margolskee RF, Kathavas P, Berg P. Epstein–Barr virus
shuttle vector for stable episomal replication of cDNA
expression libraries in human cells. Mol Cell Biol 1988; 8:
2837–2847.
14 Krysan PJ, Calos MP. Epstein–Barr virus-based vectors that
replicate in rodent cells. Gene 1993; 136: 137–143.
15 Tsukamoto H et al. Enhanced expression of recombinant
dystrophin following intramuscular administration of Epstein–Barr
virus (EBV)-based mini-chromosome vectors in mdx mice. Gene
Ther 1999; 6: 1331–1335.
16 Tu G et al. Non-replicating Epstein–Barr virus-based plasmids
extend gene expression and can improve gene therapy in vivo. J
Biol Chem 2000; 275: 30408–30416.
17 Tomiyasu K et al. Direct intra-cardiovascular transfer of beta2-
adrenergic receptor gene augments cardiac output in cardio-
myopathic hamsters. Gene Ther 2000; 7: 2087–2093.
18 Cui FD et al. Highly efficient gene transfer into murine
liver achieved by intravenous administration of naked Ep-
stein–Barr virus (EBV)-based plasmid vectors. Gene Ther 2001; 8:
1508–1513.
19 Tan Y et al. Sequential injection of cationic liposome and plasmid
DNA effectively transfects the lung with minimal inflammatory
toxicity. Mol Ther 2001; 3: 673–682.
20 Ghazizadeh S, Carroll JM, Taichman LB. Repression of retro-
virus-mediated transgene expression by interferons: implica-
tions for gene therapy. J Virol 1997; 71: 9163–9169.
21 Qin L et al. Promoter attenuation in gene therapy: interferon-
gamma and tumor necrosis factor-alpha inhibit transgene
expression. Hum Gene Ther 1997; 8: 2019–2029.
22 Li S et al. Effect of immune response on gene transfer to the lung
via systemic administration of cationic lipidic vectors. Am J
Physiol 1999; 276: L796–L804.
23 Tan Y, Li S, Pitt BR, Huang L. The inhibitory role of CpG
immunostimulatory motifs in cationic lipid vector-mediated
transgene expression in vivo. Hum Gene Ther 1999; 10:
2153–2161.
24 Yew NS et al. High and sustained transgene expression in vivo
from plasmid vectors containing a hybrid ubiquitin promoter.
Mol Ther 2001; 4: 75–82.
25 Newman PJ. The biology of PECAM-1. *J Clin Invest* 1997; **99**: 3–8.

26 Dong QG et al. A general strategy for isolation of endothelial cells from murine tissues: characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. *Arterioscler Thromb Vasc Biol.* 1997; **17**: 1599–1604.

27 Satoh E et al. Efficient gene transduction by Epstein–Barr-virus-based vectors coupled with cationic liposome and HVJ-liposome. *Biochem Biophys Res Commun* 1997; **238**: 795–799.

28 Wilson JB, Bell JL, Levine AJ. Expression of Epstein–Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. *EMBO J* 1996; **15**: 3117–3126.

29 Hammerschmidt W, Sugden B. Genetic analysis of immortalizing functions of Epstein–Barr virus in human B lymphocytes. *Nature* 1989; **340**: 393–397.

30 Tomkinson B, Robertson E, Kieff E. Epstein–Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J Virol* 1993; **67**: 2014–2025.

31 Kaye KM, Izumi KM, Kieff E. Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc Natl Acad Sci USA* 1993; **90**: 9150–9154.

32 Saeki Y, Wataya-Kaneda M, Tanaka K, Kaneda Y. Sustained transgene expression *in vitro* and *in vivo* using an Epstein–Barr virus replicon vector system combined with HVJ liposomes. *Gene Ther* 1998; **5**: 1031–1037.