Site-specific gene delivery in vivo through engineered Sendai viral envelopes

(Sendai virus/virosome/membrane fusion/gene therapy)

KOMAL RAMANI*, QUAMARUL HASSAN††, BETAPUDI VENKAIAH‡, SEYED E. HASNAIN§, and DEBI P. SARKAR*§

*Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India; and ‡Eukaryotic Gene Expression Laboratory, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi-110067, India

Communicated by Valmirni Ramalingaswami, All India Institute of Medical Sciences, New Delhi, India, July 20, 1998 (received for review February 19, 1998)

ABSTRACT Insipite of several stimulating developments in gene therapy, the formulation of a targeted gene delivery “vector” is still far from ideal. We have demonstrated the potential of reconstituted Sendai viral envelopes containing only the fusion glycoprotein (F-virosomes) in targeted delivery of reporter genes to liver cells of BALB/c mouse in vivo. The membrane fusion-mediated high efficiency of gene transfer to liver cells was ascertained following a critical evaluation of the level of the DNA, mRNA, and relevant proteins. Furthermore, the involvement of viral glycoprotein both as a unique natural ligand and as a membrane fusogen could lead to preferential transfection of parenchymal cell types of liver. The integration of transgenes in the mouse chromosomal DNA and its stable expression up to 4 mo after single i.v. administration of this gene carrier has bolstered its efficiency and novelty. Moreover, the F-virosomes did not elicit significant humoral immune response against the fusion protein in the injected animal. The findings reported here open up the possibility for considering “F-virosomes” as a promising “vehicle” for site-specific DNA delivery in gene therapy.

Despite improvements in viral and nonviral vector systems for gene therapy, current clinical trials have had limited success mainly due to inadequacies of the DNA delivery system (1). To overcome the problems associated with these vectors, we have focused our attempts on the development of an engineered Sendai viral envelope (F-virosome) that is, in essence, a hybrid vector between viral and nonviral strategies. Our findings of the specific interaction of Sendai viral fusion glycoprotein (F) with the human asialoglycoprotein receptor (ASGP-R) is of considerable importance for the development of safe and efficient hepatotropic vectors coveted for in vivo gene therapy applications (2, 3, 4). Based on interaction with ASGP-R, viral and nonviral hepatotropic gene transfer systems have been used both in vitro and in vivo. Although partial targeting to hepatocytes was achieved, the efficiency of these vectors was found to be severely impeded because of the lysosomal degradation of endocytosed ligands (5, 6, 7). Interestingly, in case of F-virosomes, the F protein acts in a bifunctional way, i.e., binding to hepatocytes followed by membrane fusion-mediated direct release of the virosomal aqueous contents to the cytoplasm of target cells (2). Having recently established F-virosome-mediated efficient delivery of foreign genes specifically into human hepatoblastoma cells in culture (3), we assessed the ability of DNA-loaded F-virosomes to affect targeted gene expression in mice after tail vein injection.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/9511886-552.00/0 PNAS is available online at www.pnas.org.

MATERIALS AND METHODS

Plasmids. The plasmid, pCIS3CAT containing chloramphenicol acetyltransferase (CAT) gene under the control of cytomegalovirus promoter, was constructed as described (3). Plasmid, pBluc (6.38 kb), containing the firefly luciferase gene under control of cytomegalovirus promoter, was derived from pCEP4-X2 luc (Stratagene). In pBluc, the luciferase-coding sequence is followed by SV40 polyadenylation sequence. These plasmids were isolated using Qiagen Megaprep kit (Chatsworth, CA).

Preparation of DNA Loaded F-Virosomes. The F-virosomes containing pCIS3CAT or pBluc DNA were reconstituted as described earlier (3). In brief, 75 μg of each plasmid was incubated with detergent solubilized fraction of Sendai virus containing its envelope devoid of hemagglutinin-neuraminidase (HN) protein. Finally, F-virosomes containing the DNA were prepared by stepwise removal of the detergent by using SM2 biobeads (Bio-Rad). Heat-treatment of loaded F-virosomes was carried out at 56°C for 30 min.

Administration of Loaded F-Virosomes and Gene Expression in Vivo. Twelve-week-old female BALB/c mice (∼18 g) were injected i.v. into the tail vein, with DNA-loaded F-virosomes (0.4 mg F protein containing 2 μg of DNA) in a final volume of 0.2 ml Tris-buffered saline (3) containing 2 mM CaCl2. Antibody response against F protein was checked as described earlier (8). (i) Reverse transcription–PCR (RT-PCR) amplification of CAT-specific transcripts: 100 μg of total RNA isolated (9) from liver was incubated with 10 units of Dnase I (Sigma) at 37°C for 15 min, and 1 μg of this RNA was reverse transcribed with 200 units of Moloney murine leukaemia virus-reverse transcriptase (GIBCO/BRL). CAT-specific transcripts were amplified by 35 cycles of RT-PCR (1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and final extension, 1 min at 72°C) by using the antisense primer 5’-TTA CGC CCC GCC CTG CCA-3’ from the 3' end of CAT and the sense strand primer, 5’-ACC GTT GAT ATA TCC CAA TGG-3’, from a sequence 27 nucleotides downstream of the 5’ end of CAT. The RT-PCR products were electrophoresed on 1.2% agarose gel and transferred to nylon membrane. A 1.5 kb Xhol/SmaI CAT gene fragment derived from the plasmid pCIS3CAT was labeled with [α-32P]dCTP by using random primer-labeling technique. The above blots were subsequently hybridized with this probe. (ii) Detection of CAT protein by ELISA: subcellular fractionation of various organs was carried out as de-

Abbreviations: F, fusion glycoprotein; ASGP-R, asialoglycoprotein receptor; CAT, chloramphenicol acetyltransferase; HN, hemagglutinin-neuraminidase; RT-PCR, reverse transcription–PCR.

| Vol. 95, pp. 11886–11890, September 1998 | Medical Sciences | 11886 |
scribed (2). In brief, the organs were placed in isotonic homogenizing buffer (0.01 M Tris-HCl (pH 7.4) containing 0.25 M sucrose) and then dispersed in Potter–Elvehjem type homogenizer at 4°C. The cytosolic fraction was purified after differential centrifugation of the homogenate. The amount of CAT protein expressed in the cytosolic fraction was quantitated using the CAT ELISA kit (Boehringer Mannheim).

Detection of CAT Gene in Liver Parenchymal Cells by PCR. Parenchymal cell (hepatocyte) separation was carried out following a standard procedure (10). To summarize, the perfused liver was excised from the animal, cut into small pieces, and washed thoroughly. The liver pieces were incubated at 37°C for 15 min in Collagenase A- (Boehringer Mannheim) containing buffer. The resulting liver mass was filtered through a nylon mesh, and the filtrate was centrifuged at 400 rpm for 10 min at 4°C to obtain the pellet-containing hepatocytes. The supernatant containing non-hepatocytes was treated with 0.2% pronaseE (Sigma) to digest the contaminating hepatocytes completely (11). The non-hepatocyte pellet was obtained by centrifuging the pronase-treated supernatant at 1,300 rpm for 10 min at 4°C. Both of these cell types were washed three times with ice-cold PBS containing 5 mM EDTA, and the cell pellets were processed for subcellular fractionation as described above. One microgram of total DNA isolated (9) from each subcellular fraction was subjected to PCR by using 1.25 units of AmpliTaq DNA polymerase (Perkin–Elmer). A 633-bp fragment of CAT gene was amplified by 35 cycles of PCR (using the conditions mentioned above). The PCR products were visualized on a 1.2% agarose gel.

Measurement of Luciferase Activity in Parenchymal and Nonparenchymal Liver Cells 24 h After Injection of pBVluc Loaded F-Virosomes. After separation, these cells were washed twice with Tricine-buffered saline and suspended in the same. For luciferase assay, the Triton X-100 solubilized cell lysate (from 1 x 10^7 cells) was mixed with luciferase assay buffer containing 1 mM luciferin (Boehringer Mannheim) (12). Luciferase activity (in mV) was measured in a 1250 Luminometer (BIO-Orbit, Finland) as described earlier (13).

State of Targeted DNA Associated with Persistent Gene Expression. Total DNA was extracted from parenchymal cells, 1, 15, 30, and 60 days after injection. The chromosomal DNA was separated from episomal DNA by gel filtration using Sephacyl S-1000 beads (14). Both the DNA fractions were ethanol precipitated and finally dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The chromosomal DNA was made free of any contaminating episomal DNA by electrophoresis on a 1% low melting point agarose gel (9). After electrophoresis of the chromosomal DNA (undigested) on 0.7% agarose gel, the blots were hybridized with 1.5-kb CAT gene fragment. Purified chromosomal DNA digested with various restriction enzymes (New England Biolabs) was hybridized as described above. The chromosomal and episomal fractions were analyzed by PCR using CAT-specific primers. The PCR cycles were as follows: initial denaturation, 94°C for 10 min; denaturation, 94°C for 1 min; annealing, 60°C for 1 min; extension, 72°C for 1 min; and final extension, 72°C for 10 min. After 35 cycles, the products were electrophoresed on 1.2% agarose gel and transferred to nylon membrane, and the blot was hybridized with 1.5-kb CAT gene fragment.

RESULTS

CAT Gene Expression in Vivo After Administration of DNA-Loaded F-Virosomes. The CAT gene expression was

![Fig. 1. CAT gene is expressed in a dose-dependent fashion. Livers from injected mice were subjected to subcellular fractionation 24 h after injection, and the amount of CAT protein expressed per milligram of cytosolic protein was determined by CAT ELISA. Each point is an average of four animals.](image)

![Fig. 2. Tissue-specific expression of CAT gene 24 h after injection. (A) RT-PCR amplification of CAT mRNA from various organs using CAT-specific primers followed by hybridization with 1.5-kb-labeled CAT gene probe. Lanes 1, 2, 3, and 4 show RT-PCR products from liver, lung, kidney, and spleen, respectively; Lanes 1'–4', corresponding samples from mice injected with heat-treated loaded F-virosomes; Lanes 1''–4'', corresponding samples from mice injected with free DNA. Positions of 1-kb ladder (GIBCO/BRL) is shown on left. (B) The amount of CAT protein in cytosolic extracts of various organs was determined by CAT ELISA. Each point represents mean value (± SD) from three animals. E, experimental; HC, heat control; FD, free DNA.](image)
assessed both at the level of mRNA and of protein. Maximum CAT expression was achieved in the liver at a dose of 2 μg of DNA loaded in F-virosomes (Fig. 1). RT-PCR analysis of total RNA from various organs confirmed the presence of CAT-specific transcripts in mouse liver (Fig. 2A). Other organs analyzed, i.e., lung, kidney, and spleen, did not express significant CAT transcripts except a very faint RT-PCR signal in case of lungs. CAT gene expression was not detected in tissues like heart, muscle, brain, lymph nodes, skin, and tail. Heat-treated virosomes and the free DNA controls also did not exhibit any CAT-specific transcripts. The relative expression of CAT protein in the liver was comparable with mRNA levels by using the same category of F-virosomes (Fig. 2B).

The expression of both mRNA and protein in mouse liver persisted over a period of 60 days (Fig. 3A and B), pointing to the overall efficiency of this delivery system. The expression also was detected until 120 days (Fig. 3B) with no F-specific humoral antibody response from 10 days onward (Fig. 3C). Moreover, the animals remained healthy and active during the experiments.

Fusion-Mediated Uptake of pCIS3CAT DNA by Liver Parenchymal Cells. Experiments were designed to determine the cellular localization of the input DNA. Total DNA from various subcellular fractions of these cells was analyzed by PCR using CAT-specific primers, that amplify a 633-bp fragment of CAT gene. After 2 h after injection of loaded F-virosomes, plasmid DNA was detected only in the cytosolic fraction (Fig. 4). After 6 h, majority of the DNA was localized in the nucleus and was detected mainly in this compartment until 24 h. No DNA was detected in the lysosomal/mitochondrial fraction at any time point after injection. In case of the corresponding heat-treated virosomes, the delivered DNA persisted mainly in the lysosomal/mitochondrial fraction until 12 h and finally could not be detected in any fraction after 24 h. PCR-amplified product was not detected from any subcellular fraction of non-parenchymal cells or after injection of free DNA.

Cell Type-Specific Luciferase Gene Expression in Mouse Liver. The luciferase activity was found to be 8–10 times greater in parenchymal cells compared with the non-parenchymal counterparts. Moreover, the luciferase expression in parenchymal cells from experimental mice was 3–4 times more than that of the corresponding heat controls. In case of non-parenchymal cells, experimental and heat control exhibited little difference in luciferase activity (Fig. 5). The purity of the cell preparations was monitored by pronase susceptibility and glass slide attachment of the non-parenchymal cells (15).

Integration of the Delivered DNA in the Chromosome of Hepatocytes. To determine the state of targeted DNA in hepatocytes after administration of loaded F-virosomes, purified chromosomal DNA was hybridized to labeled 1.5-kb CAT gene fragment derived from pCIS3CAT. No hybridization signal was detected from purified chromosomal DNA 1 day after injection; however, chromosomal DNAs from hepatocytes 15, 30, and 60 days after injection, contained high molecular weight sequences that hybridized with the CAT gene probe (Fig. 6A). Chromosomal DNA from mock-injected mice and Herring sperm DNA (GIBCO/BRL) and DNA from non-parenchymal cells failed to show any hybridization signal.

To establish the nature of integration of the DNA in mouse chromosome, purified genomic DNA from mouse liver 60 days after injection, was digested with various sets of restriction enzymes and the resulting fragments were analyzed after hybridization with 1.5-kb CAT gene probe. Purified chromosomal DNA, isolated from CAT-injected animals and restricted with different sets of enzymes, generated CAT-positive fragments of lower size than that of the linearized plasmid (6.7 kb) and a few hybridizable fragments greater in size than the linearized plasmid (Fig. 6B).

Because these band patterns do not correspond to those obtained after digestion of pCIS3CAT (data not shown), this suggests the possibility of random integration of the delivered plasmid in the mouse chromosome. The integration status was further confirmed by PCR analysis of the purified chromosomal and episomal fractions by using CAT-specific primers. Twenty four hours after injection, amplified CAT gene fragment (633 bp) was detected only from the episomal fraction but no such product was obtained from the corresponding chromosomal fractions. On the other hand, 15, 30, and 60 days
after injection, amplified products were detected only from the chromosomal fractions but not from the corresponding episomal fractions. The possibility of nonspecific PCR amplification was ruled out from appropriate negative controls (Fig. 6C). The copy number of the plasmid integrated in the genome was found to range from ~100 to 300 copies per cell as calculated from slot blot hybridization of chromosomal DNA from hepatocytes 60 days after injection.

**DISCUSSION**

The art of natural ligand-receptor interaction and Sendai viral F glycoprotein-catalyzed membrane fusion has been exploited in the construction of a safe and efficient vehicle (F-virosome) for site-specific gene delivery to mouse liver cells in vivo. The F glycoprotein is known to possess Le\(^x\) (Gal\(^\beta\)1–4[Fuc\(^\alpha\)1–3]GlcNAc-) terminated biantennary oligosaccharides (16). The Le\(^x\) moiety possesses much higher affinity for ASGP-R present on hepatocytes. This property is considered to be a prerequisite of targeted delivery systems (17). The liver is an ideal organ for transfection of a gene whose product is secreted into the circulation and is important for systemic gene therapy for several inherited diseases. Moreover, the nonpathogenicity of Sendai virus to humans and its little immunogenicity have together increased the prospects of F-virosomes in gene therapy (18, 19). The results presented here by using two reporter genes independently are highly supportive of this notion.

It has been shown earlier that heat-treated loaded F-virosomes deliver genes to the lysosomal compartment of the cell after the receptor-mediated route of entry (3). The higher efficiency of fusion-mediated delivery over receptor-mediated entry is very much pronounced below 3 \(\mu\)g of DNA dose (Fig. 1). The hepatotrotopic nature of this gene carrier is revealed clearly both in terms of mRNA (Fig. 2A) and protein levels (Fig. 2B). More striking is the sustained nature of gene expression (Fig. 3A and B). These features represent a instance of targeted and stable gene expression in vivo with no detectable adverse immune effects (Fig. 3C). Recently, attempts have been made to use liganded cationic-liposomes for transgene expression in vivo. Besides the known risk of toxicity of cationic lipids and the transient expression of CAT gene, the efficiency of this system is ~10–12 times less (20) than that of the F-virosomal delivery. In spite of a sustained insulin gene expression through Sendai virus-liposome complex, this system lacks cell-type specificity and may have undesirable side effects because of the presence of the sialic acid-binding protein (HN) of viral origin (19). The “Trojan Horse” strategy of reconstituted Sendai virus envelope in the field of drug delivery and gene therapy as reviewed earlier (21) also suffers from the lack of target specificity. Moreover, attempts have been made to deliver preproinsulin I gene through lactosylceramide containing liposomes in vivo. These vesicles are known to be internalized through ASGP-R on liver cells (11). The transient expression (until 4 h) resulting from these experiments corroborates with the endocytotic uptake of these loaded liposomes (leading to the lysosomal degradation of the transgene) in contrast to the fusion-mediated delivery through F-virosomes. Besides this, the known Le\(^x\) type of ligand present on F-virosomes confers it with the added novelty of selective targeting to selective cell types in vivo over the existing modes of DNA targeting.

The F-virosome-mediated, targeted cytosolic gene delivery in vivo to liver parenchymal cells is apprehended from the cytosolic localization of plasmid DNA at 2 h after injection and its absolute nuclear localization 6 h after injection onward (Fig. 4).
F-virosomes (3). The overall efficiency of this delivery mode also is reflected from the absence of any detectable PCR signal upon i.v. administration of free DNA. This is probably the first systematic demonstration of intracellular trafficking of a foreign gene administered in vivo. Another strong evidence of this membrane fusion-mediated targeted gene delivery is the preferential expression of luciferase gene in the parenchymal cell-types of mouse liver (Fig. 5). The persistent nature of transgene expression in hepatocytes may be explained from the stable integration of CAT gene in the mouse chromosome (Fig. 6). It is pertinent to note that sustained gene expression does not appear to be affected by the random nature of integration of pCIS3CAT DNA (Fig. 6B). The F-virosomes, being at the interface of viral and nonviral vectors, are adequately safe in comparison to their nonhybrid counterparts (1). These attributes highlight the potential of this gene carrier for targeted delivery of therapeutic genes in vivo.

We thank Dr. Prasanta K. Ghosh and Mr. Sudip Ghosh for many helpful suggestions. We are grateful to the Department of Biotechnology (DBT) (project no. BT/R&D/15/14/94), Council of Scientific and Industrial Research (project no. 60(0019)/96/EMR-II), and University Grants Commission, Government of India for financial support. The Eukaryotic Gene Expression laboratory was supported by core grants from the Department of Biotechnology.