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Herpes simplex virus as a model vector system for gene therapy in renal disease

Betsy C. Herold, Daniel Marcellino, Glendie Marcelin, Patricia Wilson, Christopher Burrow, and Lisa M. Satlin

Mount Sinai School of Medicine, New York, New York, USA

Herpes simplex virus as a model vector system for gene therapy in renal disease. The past decade has been marked by significant advances in the application of gene transfer into living cells of animals and humans. These approaches have been tested in a few animal models of inherited and acquired renal diseases, including carbonic anhydrase II deficiency [1] and experimental glomerulonephritis [2, 3]. Gene transfer into proximal tubular cells has been successfully accomplished by intrarenal arterial infusion of a liposomal complex [4] or an adenoviral vector [5]. Tubular cells from the papilla and medulla have been selectively transduced by retrograde infusion into the pelvicalyceal system of an adenoviral vector containing a reporter for β-galactosidase [5]. Although the results of these initial studies are promising, further studies to optimize viral vectors, maximize gene delivery, minimize side-effects, and develop cell-specific and long-term regulated gene expression are critical to the success of gene therapy targeted to specific compartments of the kidney. Our recent efforts have focused on defining the cellular pathways responsible for viral entry and infection into renal epithelial cells using herpes simplex virus (HSV) as a model vector. We anticipate that a solid understanding of the basic scientific principles underlying viral entry and gene expression into specific populations of renal cells will facilitate the design of successful therapeutic viral-based gene transfer strategies.

CANDIDATE VIRAL VECTORS

Optimal viral vectors for gene delivery should exhibit cell tropism for target cell type, high transduction efficiency, and little or no cytotoxicity and should elicit minimal or no immune response. Retroviruses permit efficient and stable transduction. These vectors are advantageous because they do not induce an immune response. However, random integration of the delivered gene into dividing cells raises concerns about possible insertional mutagenesis and a risk of malignant transformation. In organs with a low mitotic index, such as the mature kidney, retroviral vectors may also have limited efficiency of transduction. Adenoviral vectors can transduce a variety of cell types but are limited by transient expression of the therapeutic gene; the gene remains episomal and does not integrate into the host cell genome. Repeated applications could overcome this problem but are limited by the immune and inflammatory responses that the virus induces. Adeno-associated virus (AAV), a parvovirus, offers several advantages in that it lacks immunogenic viral proteins, can efficiently transduce nondividing cells, and integrates into the host genome at a specific site within human chromosome 19q [6]. The major disadvantages of this system are the restricted insert size the vector can accommodate (approximately 4.4 kB), difficulties in producing sufficient purified virus for gene therapy, and loss of site-specific integration with recombinant vectors developed for gene therapy. Chimeric viral vectors are now being developed that incorporate the favorable attributes of two different viral vectors.

HSV has received little attention in the arena of gene therapy for renal disease. Rather, the focus has been on the use of HSV in suicide gene therapy of tumors and its role as a vector for delivery of genes to the nervous system. However, distinct advantages of HSV as a vector suggest that further exploration of its potential role in treating renal diseases is warranted. Possibly the greatest advantage of HSV as a vector is its large genome size (approximately 150,000 bp), which could readily accommodate up to 30 to 40 kb of foreign genetic material [7, 8]. This would allow simultaneous delivery of multiple genes or a very large gene, which may be important in some disease states such as autosomal polycystic kidney disease caused by mutations in the very large protein polycystin 1 (approximately 460 kD), the product of the PKD1 gene [9, 10]. Moreover, HSV grows easily in cell culture and replicates to high titers (10^8 to 10^9), facilitating production of sufficient stocks for therapeutic purposes. The virus is well characterized with a genome that is maintained as a concatemerized circular or linear episome in infected cells. HSV readily infects epithelial cells, including renal tubular epithelia, as shown later in this article.

Two different approaches have been employed in the

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development of HSV as a viral vector for gene delivery: recombinant vectors and amplicons. Recombinant vectors have deletions in one or more essential viral gene(s). These vectors are replication defective and can establish a productive infection only on complementing cell lines that express the corresponding deleted essential viral gene(s) in *trans*. The duration of gene expression in renal cells infected with such vectors and whether repeated applications of such HSV vectors would be limited by the immune response are not known.

An alternative strategy is the HSV amplicon system. This approach involves introducing the gene(s) of interest into a plasmid containing the HSV origin of replication and packaging signal. This construct is then transfected into a cell line, which is superinfected with helper virus. The helper virus provides replication and packaging functions in *trans*, enabling the amplicon to be packaged into mature viral particles. Several different helper viruses have been developed, and recent modifications have resulted in increased yields of packaged vectors free of contamination from helper-virus [11]. Because HSV DNA replication occurs via a rolling circle mechanism, amplicon vectors contain multiple copies of the plasmid. Since these vectors contain no virus-encoded genes, they have the potential to provide long-term gene expression in transduced cells and to induce little or no immune response.

Selection of an optimal viral vector system for a specific gene therapy should take into consideration the features described previously here (for example, genome size, ability to infect dividing or nonmitotic cells, whether DNA integrates or remains episomal, and propensity to induce a host cell response), as well as the pathway of viral entry. The importance of the latter has only recently been recognized and has been underscored by experiences with adeno-associated virus (AAV) vectors for cystic fibrosis replacement gene therapy. Recent studies have shown that the apical surface of human airway epithelia is resistant to infection by AAV2, presumably because of a lack of sufficient heparan sulfate (HS) proteoglycans on the apical surface, an important receptor for AAV2 binding and entry [12, 13]. This has led to a focus on other serotypes of AAV, such as AAV5, which appear to adhere to cells in an HS-independent fashion. In addition, levels of transduction for recombinant AAV vectors vary depending on cell type, with poorly differentiated cells transducing more efficiently than well-differentiated epithelial cell types [13]. The efficiency of AAV-mediated ex vivo gene transfer is limited to regions of epithelial injury and preferentially targets basal-like cells [14, 15]. Together, these studies highlight the importance of defining, at a molecular level, the pathway of entry for candidate viral vectors and testing viral vectors in primary culture systems.

HSV ENTRY INTO RENAL EPITHELIAL CELL LINES

Our studies focus on the potential role of HSV as a viral vector for gene therapy for renal diseases by investigating how the virus attaches to and invades target renal cells. Using Vero cells, a cell line derived from the kidney of a normal adult African green monkey, the pathway of HSV-1 entry has been partially characterized. Briefly, invasion of Vero cells in culture by HSV requires binding of virus to receptors on the cell surface, followed by fusion of the virion envelope with the cell plasma membrane. This process is complex and involves multiple interactions between viral envelope glycoproteins and cell surface components. The initial interaction is binding of viral envelope glycoproteins gC and/or gB to cell surface HS [16–18]. The evidence includes the following key observations. Cells devoid of HS (but not other glycosaminoglycans) because of enzymatic treatment or genetic mutation have greatly reduced the numbers of receptors for HSV binding. These cells are poorly infected by HSV but are unaltered in their susceptibility to other viruses, such as vesicular stomatitis virus [19, 20]. Soluble heparin, which is structurally similar to HS, inhibits the binding of HSV to cells [20]. In contrast, chondroitin sulfate, a different glycosaminoglycan, fails to inhibit viral binding or infection. The viral envelope glycoproteins gC and gB bind selectively and independently to heparin-sepharose columns under physiological conditions [16]. Deletion of gC alone, but not gB, reduces, but does not abolish, specific viral binding activity, whereas deletion of both gC and gB abolishes viral binding [16, 17]. Together, these results suggest that initial binding of HSV-1 to Vero cells is mediated principally by interactions of viral envelope glycoprotein gC with cell surface HS moieties. Thus, although gC is nonessential in cell culture, viral vectors deleted for this glycoprotein would be predicted to exhibit reduced binding activity and efficiency of gene transduction.

Expression of HS proteoglycans is cell and tissue-type specific and is developmentally regulated [21–25]. The HS chain consists of alternating residues of an amino sugar and a uronic acid. As the chains polymerize, they undergo various sulfation and epimerization reactions that result in structurally quite heterogeneous sequences differing in sequence, chain length, and degree, extent, and pattern of sulfations. These structurally diverse sequences may be differentially expressed on various cell types and on the apical or basolateral surfaces of polarized epithelial cells. Knowledge of the specific HS sequence that serves as the receptor for initial viral attachment and its relative expression on target cells is critical to the success of viral gene therapy.

Following the initial HS attachment step, glycoprotein D (gD) binds to cellular coreceptors. Several candidate

gD coreceptors have been identified, including a member of the tumor necrosis factor (TNF) receptor family called herpes virus entry mediator A (HveA), and two members of the immunoglobulin superfamily, termed HveB (nectin2α) and HveC (nectin-1) [26–29]. Most recently, a unique HS sequence has been shown to serve as a HSV-1–specific gD coreceptor [30]. These different gD coreceptors may be differentially expressed on various cell types, and the redundancy of the coreceptors may explain, in part, the diverse tropism of HSV. The specific gD coreceptor expression on human renal epithelial cells has not yet been characterized.

How the interactions of gC and gB with HS sequences and gD with its coreceptors trigger penetration is not known. Penetration is mediated by a pH-independent fusion of the virion envelope with the cell plasma membrane and requires the concerted action of gB, gD, and hetero-oligomers of gH-gL [31].

We recently explored the possibility that binding of virus to cell surfaces might trigger activation of a signal transduction pathway and facilitate viral entry [32]. This hypothesis emerged from the observations that other microbial receptor–ligand interactions trigger signal transduction pathways, candidate coreceptors for gD are linked to these pathways, and the common role of HS is as a receptor for growth factors (which act via signal transduction pathways) and HSV. Using immunoprecipitation and Western blotting, we found that at least three cytoplasmic Vero cell proteins become tyrosine phosphorylated within five minutes after exposure to either serotype of HSV (Fig. 1) [32]. However, no phosphorylation is detected when cells are exposed to a mutant virus deleted in gL (but expressing gC, gB, and gD at the cell surface), which presumably binds to HS and gD coreceptors (discussed later here). Phosphorylation is restored when the gL-deletion virus is grown on a complementing cell line. These studies suggest that the interactions of gC or gB with HS and gD with its coreceptor are not sufficient to induce activation of this pathway.

One possible mechanism that could explain how activation of signal transduction pathways might facilitate viral entry is through changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and alterations in actin microfilaments. To test this hypothesis, Vero cells were loaded with fura-2-AM for 30 minutes, superfused in the loading chamber with phosphate-buffered saline (PBS) for 15 minutes, and then exposed sequentially to PBS buffer or HSV-1 at a multiplicity of infection of 5 pfu/cell. For these studies, we selected a virus deleted in an essential viral gene, gL, as a prototypic candidate viral vector for gene therapy. The viral strain HSV-1(KOS)gL86 is a generous gift of P. Spear (Northwestern University, Chicago, IL, USA). The deletion of gL results in mutant virions that can bind to cells but fail to penetrate [33]. For these studies, stocks of KOS-gL86 harvested from complementing cells were prepared; this virus is phenotypically wild type but genotypically gL deleted. The stock harvested from complementing gL-expressing 79VB4 cells (gift of P. Spear) had a titer of approximately 10^9 pfu/mL on 79VB4 cells; no plaques were detected on Vero cells. Changes in $[Ca^{2+}]_i$ were measured in individually identified Vero cells (5 to 6 cells per experiment) by digital ratiometric imaging (340 nm/380 nm); a calibration was performed at the conclusion of each experiment with EGTA/AM and ionomycin, according to standard methodology [34]. Results from a representative experiment are shown in Figure 2A. Similar results have been obtained in 15 experiments, although there is individual cell variability in the response. Exposure to HSV-1 results in a rapid increase in $[Ca^{2+}]_i$, which peaks within 40 seconds and rapidly returns to baseline.

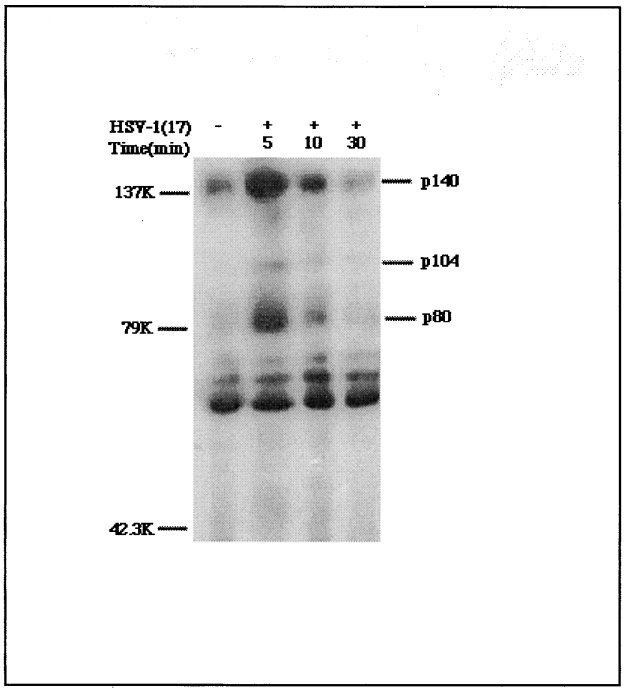

Fig. 1. Stimulation of cellular protein tyrosine phosphorylation after exposure to HSV-1. Vero cells were mock infected or exposed to 10 PFU/cell of HSV-1(17) for 5, 10, or 30 minutes. Cell lysates were prepared and analyzed by immunoprecipitation with PY20-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoprecipitated proteins were detected by Western blotting with PY20, an anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY, USA). Molecular weight standards are indicated on the left. The bars on the right identify the three cellular proteins (p80, p104, and p140) that are tyrosine phosphorylated after exposure to HSV-1. Adapted from Qie et al [32].

To determine whether the physical binding of virus to cells is sufficient to induce the changes in $[Ca^{2+}]_i$, Vero cells were loaded with the fura-2-AM dye and were exposed to the KOS-gL86 virus that had been grown on noncomplementing cells. This virus is both phenotypically and genotypically gL deleted. Virus harvested from

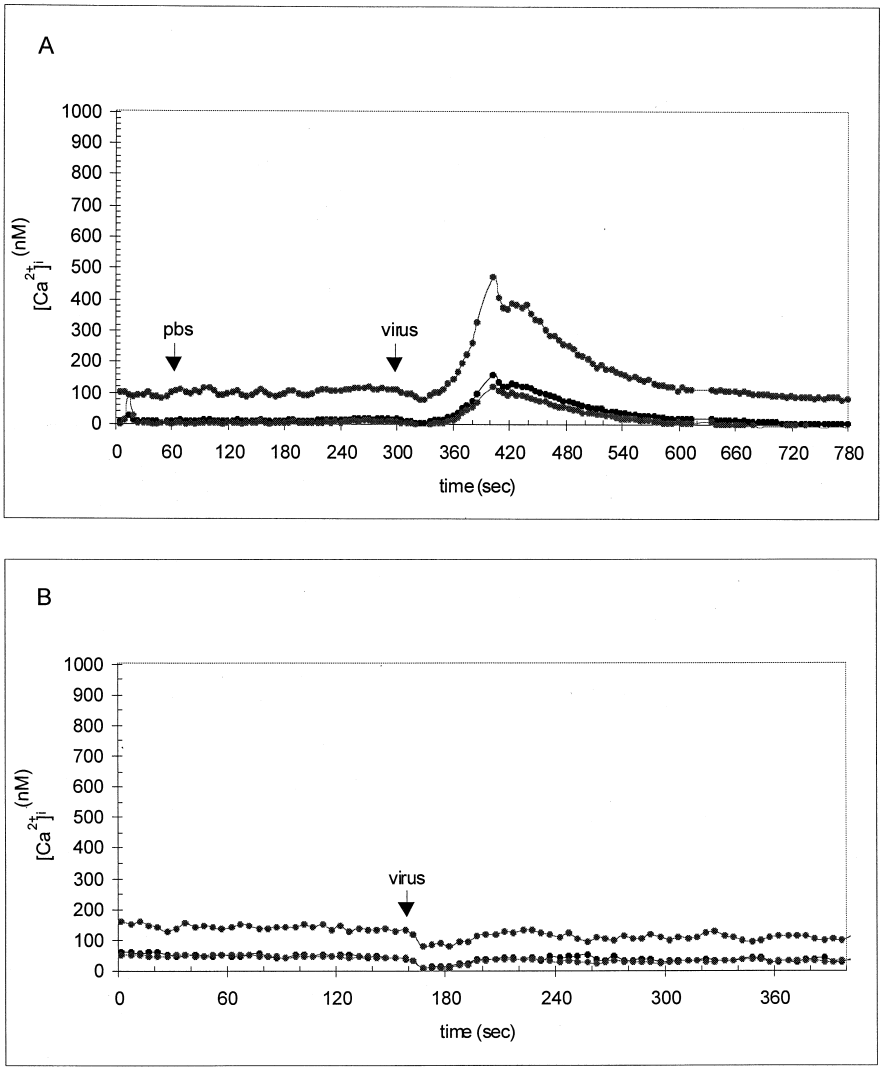

Fig. 2. Effect of viral entry on intracellular Ca^{2+} ($[Ca^{2+}]_i$) in Vero cells. Cells were loaded with the fluorescent Ca^{2+} indicator fura-2, and their fluorescence intensity ratios (340 nm/380 nm) were monitored before, during, and after exposure to either PBS or virus. (*A*) Cells were exposed to HSV(gL86) harvested from complementing cells (phenotypically wild type) and (*B*) to HSVgL86 harvested from Vero cells, rendering the virus able to bind but unable to penetrate the cells.

noncomplementing Vero cells had a titer of $<10^3$ pfu/mL on both Vero and 79VB4 cells, reflecting a low level of recombinants. The relative concentration of viral particles was determined by comparing the optical densitometry of the gD band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, as previously described [32]. Fura-2-treated cells were exposed to equivalent particle numbers, and the $[Ca^{2+}]_i$ was monitored. As shown in Figure 2B, there was no increase in $[Ca^{2+}]_i$ following exposure of cells to noncomplemented virus. This suggests that binding alone is not sufficient to induce changes in $[Ca^{2+}]_i$.

Studies are currently ongoing to determine whether influx of extracellular Ca^{2+} and/or release of Ca^{2+} from endoplasmic reticulum stores serve as the source of increase in $[Ca^{2+}]_i$ and the importance of this pathway in viral entry. $[Ca^{2+}]_i$ is required for fusion of a wide variety of biological membranes, including the fusion of some enveloped viruses with cell membranes [35]. For example, binding of human immunodeficiency virus type 1 to the CD4 molecule induces phosphatidylionistol-3-kinase [36, 37]. $[Ca^{2+}]_i$ also appears to play a role in Epstein-Barr virus penetration and human cytomegalovirus cell fusion [38, 39]. We speculate that changes in $[Ca^{2+}]_i$ may play an important role in facilitating HSV viral envelope-cell fusion, perhaps through rearrangements of actin microfilaments. Understanding this pathway in human renal epithelial cells should facilitate the development of optimal HSV vectors for gene therapy.

HSV ENTRY INTO HUMAN RENAL EPITHELIAL CELLS

We have begun to elucidate the viral and cellular components and pathways critical for HSV invasion of renal epithelial cells. To validate whether the results obtained in cell lines will be applicable to human cells and tissue,

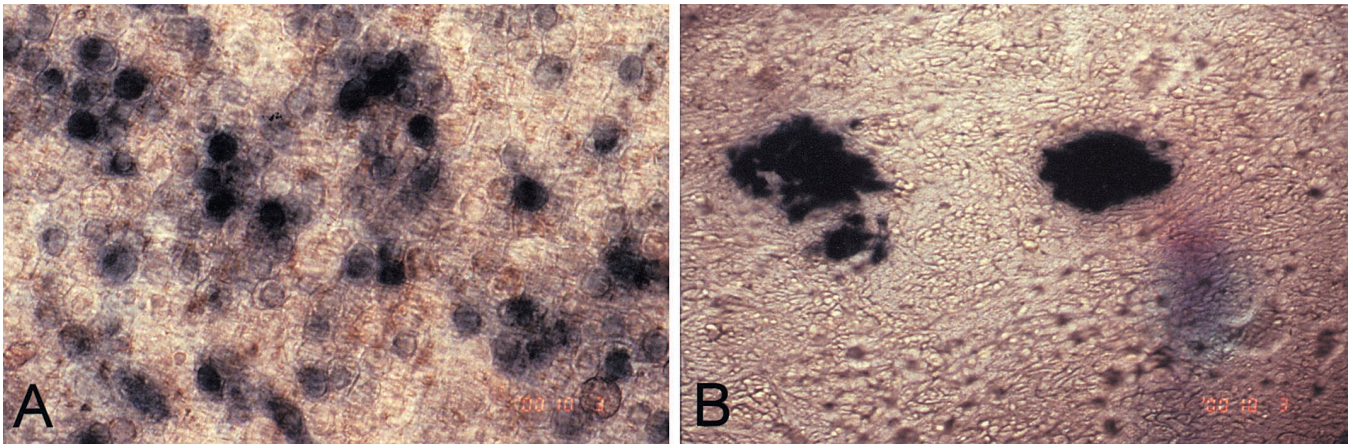

Fig. 3. Human renal tubular epithelial cells are susceptible to HSV-1 infection. Conditionally immortalized human renal collecting duct cells were infected overnight with serial dilutions of HSV-1(17)(dUTPase) from the apical (*A*) or basolateral (*B*) compartments. Monolayers were fixed and permeabilized and stained for β-galactosidase expression.

we are studying the pathway of HSV viral entry into specific populations of human renal tubular epithelial cells.

We found that HSV-1 readily infects immortalized normal human renal tubular epithelial cells (Fig. 3). Conditionally immortalized human renal proximal tubule (PST), collecting tubule (CT), and thick ascending limb (TAL) epithelia were plated on collagen-coated Transwell (Costar, Cambridge, MA, USA) membrane filters, grown to 80% confluence in segment-specific media at the permissive temperature 33°C and transferred to 37°C for 10 days to maximize differentiation [40–42]. Cells were infected with HSV-1[17] (dUTPase) virus, which expresses the reporter gene β-galactosidase under control of a viral early immediate gene product (generous gift from E. Wagner, University of California, Irvine, CA, USA). Virus was added to either the apical or basolateral compartments, and infectivity was monitored for 24 hours after inoculation by staining for β-galactosidase expression (Fig. 3) [32]. All three cell types were susceptible to infection from both the apical or basolateral surface. In preliminary studies, cells were more susceptible to HSV when exposed from the apical surface. Infection was inhibited by soluble heparin, suggesting that virus entry into these cells is HS dependent. Further studies are ongoing to explore the molecular basis for differences in susceptibility to apical and basolateral infection among the three cell types. It remains to be determined whether differences in apical and basolateral expression of HS receptors, gD coreceptors, or signaling pathways correlate with susceptibility to infection.

SUMMARY

The results of the investigations summarized in this article indicate that HSV entry into renal epithelial cell lines triggers tyrosine phosphorylation of host cellular proteins and involves a signaling pathway associated with an increase in $[Ca^{2+}]_i$. HSV, a vector in which the large genome size would allow delivery of very large genes, such as those encoding PKD1, efficiently infects human renal epithelial cells. Further exploration of the mechanisms mediating viral entry is critical to the development of optimal vectors to treat diseases unique to specific renal epithelial cell types.

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Reprint requests to Betsy C. Herold, M.D., Box 1657 Mount Sinai School of Medicine One Gustave L. Levy Place, New York, New York 10029-6574, USA.
E-mail: betsy_herold@mssm.edu

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