Transcriptional Targeting of Herpes Simplex Virus for Cell-Specific Replication

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Received 13 January 1997/Accepted 19 March 1997

Tissue- or cell-specific targeting of vectors is critical to the success of gene therapy. We describe a novel approach to virus-mediated gene therapy, where viral replication and associated cytotoxicity are limited to a specific cell type by the regulated expression of an essential immediate-early viral gene product. This is illustrated with a herpes simplex virus type 1 (HSV-1) vector (G92A) whose growth is restricted to albumin-expressing cells. G92A was constructed by inserting an albumin enhancer/promoter-ICP4 transgene into the thymidine kinase gene of mutant HSV-1 d120, deleted for both copies of the ICP4 gene. This vector also contains the Escherichia coli lacZ gene under control of the thymidine kinase promoter, a viral early promoter, to permit easy detection of infected cells containing replicating vector. In the adult, albumin is expressed uniquely in the liver and in hepatocellular carcinoma and is transcriptionally regulated. The plaquing efficiency of G92A is >103 times higher on human hepatoma cells than on non-albumin-expressing human cells. The growth kinetics of G92A in albumin-expressing cells is delayed compared with that of wild-type HSV-1, likely due to aberrant expression of ICP4 protein. Cells undergoing a productive infection expressed detectable levels of ICP4 protein, as well as the reporter gene product β-galactosidase. Confining a productive, cytotoxic viral infection to a specific cell type should be useful for tumor therapy and the ablation of specific cell types for the generation of animal models of disease.

Gene therapy strategies for cancer have used various delivery methods, including viral vectors, to transfer suicide genes (21, 48) or immune-modulator genes (64, 73) to neoplastic cells. We recently developed an alternative approach that uses the inherent cytotoxic capabilities of replication-competent herpes simplex virus type 1 (HSV-1) to destroy tumor cells in vivo and in the process replicate and spread throughout the tumor (29, 42). Our studies with malignant brain tumors used mutants of HSV-1 that were unable to replicate in nondividing cells and/or were attenuated for neurovirulence (29, 41, 42, 46, 47).

To expand the clinical potential of this viral oncolytic therapeutic approach to other tumors and to avoid toxicity to normal dividing cells (endothelial, fibroblast, epithelial, etc.), it is possible to exploit the transcriptional differences between normal and neoplastic cells. The targeting of gene therapy to the appropriate tumor cell type so that normal cells are not adversely affected has been achieved at the level of physical transfer, the replicative status of the cell, or the transcriptionally regulated expression of the transferred gene (45). Tissue-specific regulatory sequences have been used to drive expression of suicide genes following retrovirus- or adenovirus-mediated gene transfer (23, 28, 39, 69, 72, 75), direct injection of DNA (76), and adenovirus-polylysine-mediated transduction (14). Rather than target expression of a specific gene product for therapy, we have chosen to target the complete lytic growth cycle of the virus. For tumor therapy, the ability to synthesize new infectious viral particles that will spread to adjacent cells throughout the tumor should amplify the cytotoxic potential of the vector.

The feasibility of confining the host range of HSV-1 to a particular cell type was tested in the following model system that uses the mouse albumin enhancer/promoter sequence (26, 55) as the cell-type-specific regulatory region. Albumin is expressed uniquely in liver and is regulated at the level of transcription initiation (74). The albumin gene contains a liver-specific promoter of about 150 bp just upstream of the cap site (24). A region from 8.5 to 10.4 kb upstream of the albumin promoter functions as an enhancer that, in combination with the albumin promoter (300 bp), drives high-level expression specifically in the adult liver of transgenic mice (55), in infected hepatocytes in vivo after delivery of recombinant retroviruses through the portal vein and partial hepatectomy (22, 36), and in human hepatoma cells after infection with recombinant adenovirus (25) or recombinant retroviruses (28, 39). The albumin enhancer/promoter was used to drive expression of the HSV ICP4 gene.

HSV genes, during a lytic infection, are expressed temporally in three major classes: immediate-early (IE or α), early (E or β), and late (L or γ) (7, 27). Progression through this growth cycle is dependent on two essential IE proteins, ICP4 and ICP27 (10, 57, 62). The ICP4 (Vmw175, IE175, IE-3, α4) gene is located in the short inverted repeat regions of the HSV genome (Fig. 1A) and encodes a 175-kDa phosphorylated nuclear protein that is the main trans activator of HSV transcription (54, 56). Mutants lacking ICP4 fail to synthesize early or late viral polypeptides but continue to express the other IE genes (10, 12, 56, 57, 78). Binding of ICP4 protein to a degenerate consensus sequence present upstream of a number of HSV-1 genes (e.g., ICP4, ICP0, latency-associated transcript, ICP8, thymidine kinase [TK], gC, and gD genes) results in either repression of IE genes or activation of E and L genes (3, 11, 19, 38, 44, 60). The negative regulation of ICP4’s own transcription occurs in a temporal fashion postinfection (p.i.).
ICP4 also acts in concert with IE proteins ICP0 and ICP27 to regulate viral gene expression (13, 15, 51). The second chimeric transgene, with the *Escherichia coli* lacZ gene under control of the HSV-1 TK promoter, was used to easily screen for recombinant viruses and identify cells containing replicating virus. TK is an E gene, and therefore lacZ expression should be dependent on synthesis of ICP4 protein (11, 16, 57, 65, 78). The chimeric transgenes (albumin enhancer/promoter-ICP4, TK promoter-\textit{lacZ}) were inserted into the HSV-1 TK gene (UL23), resulting in a 0.5-kb deletion of the TK gene and inactivation of the UL24 gene (31). HSV-1 mutants lacking TK activity grow poorly in nondividing cells (32, 35) and have been shown to be effective in brain tumor viral therapy (4, 29, 33, 35, 42).

**MATERIALS AND METHODS**

**Cells and viruses.** Hep3B (with integrated hepatitis B virus [HBV] positive), HepG2 (HBV negative), HuH7 (HBV negative), and SW480 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% heat-inactivated fetal calf serum (HyClone). MCF7 cells were grown similarly except that the medium also contained 15 mg of endothelial cell growth supplement (Becton-Dickinson) per ml, and E5 cells were grown in medium supplemented with 10% newborn calf serum (HyClone). Detroit 351 cells were grown in Eagle’s minimal essential medium supplemented with 10% newborn calf serum, 0.1% lactose hydrolysate, and 1 mM sodium pyruvate. HepG2, Hep3B, MCF7, Detroit
antibody (Zymed). Protein determinations, from lysates of scraped cells, were performed with the method of Bradford (5), using the Bio-Rad protein assay kit according to the manufacturer’s instructions and bovine gamma globulin as a standard.

RESULTS

Generation of albumin enhancer/promoter-regulated recombinant HSV-1 (G92A). We constructed an HSV-1 recombinant regulated by the albumin enhancer/promoter and termed G92A (Fig. 1A). G92A is derived from d120, an HSV-1 mutant with a deletion of the ICP4 gene (10, 12). d120 can grow only on ICP4-complementing cell lines such as E5 (10, 12). G92A was constructed by recombining sequences from plasmid pTKAL-AI14 into the TK locus of d120. Plasmid pTKAL-AI14 contains two chimeric transgenes (Fig. 1A) expressing ICP4 protein and β-galactosidase. The mouse albumin enhancer/promoter, containing upstream sequences from kb −10.5 to −8.5 fused to the proximal promoter sequences from pb −300 to +22 (55), was cloned upstream of the ICP4 coding sequence (at +178; ICP4 transcription begins at +301) and polyadenylation site. The E. coli lacZ coding sequence and SV40 polyadenylation site were inserted into the HSV-1 TK gene just downstream of the TK promoter so that β-galactosidase expression was regulated by the HSV-1 TK promoter (an early promoter). With lacZ upstream of the albumin enhancer/promoter, it is very unlikely that read-through transcription from the TK promoter could occur. The 3′ untranslated region of TK, downstream of the ICP4 transgene, overlaps the 5′ promoter region of gh (65), which should have no impact on the regulation of ICP4 expression. pTKAL-AI14 was cotransfected with d120 DNA into E5 (ICP4+) cells, and the resulting recombinant viruses were thrice plaque purified on E5 cells. Recombinant plaques stained blue with X-Gal because of the presence of the lacZ gene, grew in the presence of ganciclovir because they were tk−, and formed plaques on HepG2 cells, which express albumin.

Viruses stocks were prepared on HepG2 cells to minimize the possibility of reversion of the ICP4 deletion by recombination with the complementing ICP4 gene in E5 cells. We prepared two independently isolated recombinant stocks, G92A1 and G92A2, which were identical by all criteria tested. The DNA structures of these recombinants (Fig. 1A) were confirmed by restriction endonuclease digestion and Southern blot analysis, using probes to ICP4 (pXhoI-C), lacZ (pHCL), albumin enhancer/promoter (p2335A-1), and HSV-1 TK (pHSV106) genes (Fig. 1B). The recombinant viruses maintain the ICP4 deletions of d120 (Fig. 1B, probe pXhoI-C). In addition, they contain the ICP4 and lacZ transgene insertions (Fig. 1B, probes pXhoI-C and pHCL) in the HSV-1 TK gene, resulting in two new fragments hybridizing to the TK gene and loss of the 3.6-kb fragment from d120 (Fig. 1B, probe pHSV106).

G92A replication in human albumin-expressing cells. The ability of G92A to replicate specifically in albumin-expressing cells was examined in a single-step growth experiment (Fig. 2). Human Hep3B hepatoma cells express albumin, whereas human MCF-7 breast adenocarcinoma cells do not (28). Infection of either Hep3B or MCF7 cells with wild-type, parental HSV-1 strain KOS resulted in similar viral replication kinetics and viral yields, indicating no cell-specific effects on HSV replication. However, no viral burst (10-fold lower than input virus) was detected with G92A-infected MCF7 cells (Fig. 2). A similar lack of viral burst was also seen with G92A-infected HeLa (human epitheloid carcinoma), CAKI-2 (human renal cell carcinoma), and PC3 (human prostate adenocarcinoma) cells (data not shown). G92A-infected Hep3B cells produced a significant viral burst, less than that obtained with KOS, and...
growth of G92A was delayed and slower than that of KOS (Fig. 2).

Cell specificity of G92A growth on human cells in culture.
The ability of G92A to form plaques on a number of different human cell lines was determined to examine both the replication and spread of the virus in culture. In addition to Hep3B, both HuH7 and HepG2, which are HBV-negative human hepatoma cell lines, express albumin (1, 9, 37, 49). Human SW480 colon adenocarcinoma cells and Detroit 551 diploid fibroblast cells do not express detectable albumin (28).

All cell types were similarly sensitive to wild-type HSV-1 infection, as illustrated by the ability of HSV-1 strain KOS to form plaques on all cells tested with similar efficiencies (Fig. 3 and 4C, E, and K). Plaque morphology varied depending on the cell type (Fig. 4). The parental virus, d120 (ICP4−), formed plaques efficiently only on E5 (ICP4+) cells (Fig. 3 and 4A). A few plaques formed on some other cell types at approximately 10−5 to 10−6 the frequency on E5 cells, possibly due to the presence of revertants of d120 in the virus stock (10, 12). In contrast to wild-type HSV-1, G92A formed plaques with high efficiency only on albumin-expressing hepatoma cells (Fig. 3 and 4D and F), with an efficiency similar to that seen on E5 cells (Fig. 3 and 4B). Both independently isolated G92A stocks behaved similarly (Fig. 3). The abilities of G92A or KOS to produce plaques were the same at the three incubation temperatures tested (31.5, 34, and 37.5°C).

Albumin-nonexpressing cells were highly refractory to G92A replication, and so there was a markedly decreased plaquing efficiency on Detroit 551, SW480, and MCF7 cells (Fig. 3 and 4L, N, and P). There was a greater than 103-fold difference between plaquing ability on albumin-expressing and -nonexpressing cells. We used very broad latitude in defining plaques so that all clusters of cells demonstrating cytopathic effect (CPE) were counted, regardless of their size or extent of CPE. In many cases, staining with X-Gal (Fig. 4L, N, and P) or antibody to ICP4 (Fig. 4J) was the only way to detect these plaques (for example, compare G92A plaques with KOS plaques on albumin-expressing [Fig. 4E and F] and -nonexpressing [Fig. 4L and K] cells). The time of incubation for the G92A-infected nonexpressing cells was usually 1 to 7 days longer than the corresponding time for wild-type KOS, whereas for the albumin-expressing cells, the incubation times were similar. It is possible that at the high titers of G92A plated on nonexpressing cells, cells infected with multiple viral particles were able to overcome the block to viral replication (63, 71). The MCF7 cells consistently yielded higher titers than the other albumin-nonexpressing cells, even with the parental ICP4 deletion mutant d120, and may contain factors able to complement the ICP4 deficiency at a low level.

FIG. 3. Plaques of G92A is restricted to albumin-expressing (Alb+) human cell lines, as opposed to wild-type HSV-1 KOS, which has no cell specificity. Monolayers of E5 (ICP4+), Hep3B (Alb+), HuH7 (Alb+), HepG2 (Alb+), Detroit 551, SW480, and MCF7 cells grown in six-well dishes were infected with serial dilutions of HSV-1 strains KOS (wild type), G92A1, G92A2, and d120 (ICP4−). No consistent significant difference in titer was seen at different incubation temperatures or between Giemsa- and X-Gal-stained plates. The number of PFU was calculated from the mean number of plaques formed ± standard deviation on albumin-expressing cells (solid bars) and -nonexpressing cells (hatched bars). N is the number of separate experiments in which titers were determined (each titer is the average of plaque counts obtained from ≥2 wells, usually of two different dilutions). ∗, at the lowest possible viral dilution (usually 10−7), no plaques were detected.
Transgene expression. Substantial β-galactosidase expression was detected in cells surrounding plaques of G92A-infected albumin-expressing cells (Fig. 4B, D, and F). Infection with HSV-1 hrR3 (ICP6\(^{-}\) lac\(^{+}\) [20]) produced ICP4\(^{+}\) (Fig. 4G and I) and X-Gal\(^{+}\) (Fig. 4M and O) plaques on all cell types tested. ICP4 protein localized to the nucleus and was detected where plaques formed on G92A-infected albumin-expressing...
cells (Fig. 4H). Clusters of ICP4+ cells were sometimes seen after infection of nonexpressing cells (at approximately $10^{-3}$ the efficiency seen on albumin-expressing cells) (Fig. 4J), likely due to non-albumin-specific expression. Low levels of reversion at the ICP4 loci should have produced wild-type-sized plaques. Synthesis of ICP4 protein in the various cell types was also determined by immunofluorescence (data not shown). At 48 h p.i. (prior to the formation of plaques), ICP4+ cell clusters were detected only in G92A-infected hepatoma cultures (Hep3B and HepG2) or hrR3-infected MCF7 cells. ICP4 expression in E5 cells was detected only after infection with HSV-1 (data not shown). When ICP4 expression was examined, all plaques or clusters of cells exhibiting CPE were ICP4 immunoreactive.

The time course of ICP4 protein expression was determined by Western blotting of cell lysates from HepG2 cells infected
Expression in G92A-infected cells is presumably regulated by cellular factors, in contrast to viral factors for KOS or hrR3. The viral growth difference observed is likely due to differences in the regulation of ICP4 protein expression and its consequent downstream effects on E and L gene expression. Some of the decreased growth could be due to the deletion in the UL24 gene, which has been shown to result in smaller burst sizes (31). Other HSV-1 mutants with decreased viral burst size (hrR3 and G207) have been found to be effective in inhibiting tumor growth in vivo (46, 47).

A strategy alternative to that described in this study is to replace the ICP4 promoter of the endogenous ICP4 gene with a novel regulatory sequence. The pseudorabies virus IE180 (homolog of HSV ICP4 [79]) promoter was replaced with the inducible promoter of the drosophila HSP70 gene (17) or the bovine cytokeratin IV promoter (17). A 2- to 3-log difference in pseudorabies virus titer was seen upon comparison of the plaquing abilities of the HSP70 promoter recombinant at inducing and noninducing temperatures or the cytokeratin IV promoter recombinant in a cytokeratin-expressing bovine epithelial cell line and in a swine kidney cell line (18). A drawback to this approach for HSV is illustrated by the studies of Smith et al. (68), who generated deletions in both copies of the ICP4 promoter region in HSV-2. A mutant deleted for the entire ICP4 promoter had undetectable amounts of ICP4 mRNA in the presence of cycloheximide yet was still viable in cells that do not express ICP4 (68). The replication of this mutant could be due to the presence of promoter sequences in ori that are regulated as early or late transcripts (blocked by cycloheximide treatment); alternatively, transcript levels below those detectable may have been sufficient for viral replication. Larger deletions that included ori were not viable (68). Igarashi et al. (30) were able to generate HSV-1 mutants deleted for both ori sequences if one of the ICP4 promoters was replaced with the SV40 promoter. These recombinants grew similarly to, though more slowly than, parental ori HSV-1 (30), suggesting that the viral transcriptional program was not greatly altered by the SV40 promoter-ICP4 transgene present at the endogenous ICP4 location.

The second transgene inserted into G92A contains the E. coli lacZ gene regulated by an E HSV-1 promoter (TK). LacZ is expressed in those cells where the recombinant virus is replicating and can be easily detected by X-Gal histochemistry. Other coding sequences, such as for immune-modulatory proteins, could be placed under control of viral E and L promoters (in place of or in addition to the lacZ gene used here) such that they will be expressed only in those cells where virus is replicating. Thus, it may be possible to elicit an immune response specifically directed at tumor cells while minimizing the damage to normal tissue. HSV-1 is an especially useful vector for these purposes because of its large size (with many nonessential genes) and broad host range. Transgene insertions need not be in the TK locus. Other loci in the virus, for example, the ICP6 (ribonucleotide reductase) gene (47), may also be useful sites for recombination. ICP6 mutants retain sensitivity to acyclovir and ganciclovir, whereas TK mutants are resistant (8, 46).

These studies demonstrate the feasibility of transcriptionally targeting viral replication to specific cell types. Unlike most HSV-1 host range mutants, where viral growth is limited to specially isolated or constructed complementing cells (such as the E5 cells), the strategy described here targets normal or transformed cells and is applicable to in vivo models. In previous studies, promoter elements inserted into the HSV-1 genome were affected by the regulatory properties of the surrounding HSV-1 sequences (53, 61). One of the reasons that G92A is successfully regulated may be due to the lack of
normal IE gene expression. In the absence of ICP4 protein, the HSV-1 genome is relatively inactive transcriptionally, except for the other IE genes (10), and therefore recombinant sequences may be less prone to the normally strong positional effects of the viral genome. In addition, regulated expression of the ICP4 transgene in G92A does not have to be maintained for extended periods of time as is the case with standard gene delivery vectors, but just long enough to initiate and maintain viral replication.

G92A would likely be of limited application in humans due to the expression of albumin in both normal hepatocytes and hepatoma cells. However, G92A may be useful in experimental models of hepatocellular carcinoma. This is because the vector is TK− and should grow poorly in nondividing adult hepatocytes (which express high levels of albumin). In a preliminary experiment, we found that inoculation of G92A (10⁵ PFU) into the livers of young BALB/c mice was not lethal. HSV-1 TK− mutants have been used as viral therapy for brain tumors (4, 29, 33, 35, 42), where glioma cells are dividing and the surrounding neurons and glia are nondividing. This growth differential is less pronounced in human hepatocellular carcinoma because of its association with chronic liver disease and hepatocyte regeneration (77). G92A might also prove useful for the generation of animal models of hepatocyte necrosis, a pathology associated with many nonmalignant diseases of the liver.

Other, more tumor-specific enhancers and/or promoters are the obvious regulatory regions to be attempted next. Examples include the tyrosinase promoter and melanoma cells (75), the obvious regulatory regions to be attempted next. Examples include the tyrosinase promoter and melanoma cells (75), the heat-shock gene HSP70 on the growth and virulence of pseudorabies virus. Vet. Microbiol. 33:43–44.


