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Reduction in Severity of a Herpes Simplex Virus Type 1 Murine Infection by Treatment with a Ribozyme Targeting the U_L20 Gene RNA

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Hammerhead ribozymes were designed to target mRNA of several essential herpes simplex virus type 1 (HSV-1) genes. A ribozyme specific for the late gene U_L20 was packaged in an adenovirus vector (Ad-U_L20 Rz) and evaluated for its capacity to inhibit the viral replication of several HSV-1 strains, including that of the wild-type HSV-1 (17syn+ and KOS) and several acycloguanosine-resistant strains (PAAR5, tkLTRZ1, and ACGr4) in tissue culture. The Ad-U_L20 Rz was also tested for its ability to block an HSV-1 infection, using the mouse footpad model. Mouse footpads were treated with either the Ad-U_L20 Rz or an adenoviral vector expressing green fluorescent protein (Ad-GFP) and then infected immediately thereafter with 10^4 PFU of HSV-1 strain 17syn+. Ad-U_L20 ribozyme treatment consistently led to a 90% rate of protection for mice from lethal HSV-1 infection, while the survival rate in the control groups was less than 45%. Consistent with this protective effect, treatment with the Ad-U_L20 Rz reduced the viral DNA load in the feet, the dorsal root ganglia, and the spinal cord relative to that of the Ad-GFP-treated animals. This study suggests that ribozymes targeting essential genes of the late kinetic class may represent a new therapeutic strategy for inhibiting HSV infection.

Effective ribozyme inhibitors can be identified through a combination of cell-free kinetic testing and cell-based functional assays.

HSV-1 carries more than 80 genes, which are organized into three temporal expression classes, immediate early (alpha), early (beta), and late (gamma) (30). The goal of this study was to evaluate the effects of targeting essential HSV-1 lytic gene RNAs for degradation, using hammerhead ribozymes. Since even the most efficient ribozyme would not be predicted to cleave 100% of the targeted viral RNA, we chose to identify the viral lytic gene mRNAs whose cleavage by ribozymes would result in a significant reduction in viral yield. We therefore designed hammerhead ribozymes to cleave mRNAs of four HSV-1 essential genes (ICP4, U_L20, U_L30, and U_L54), and these ribozymes were tested for their abilities to cleave the RNA targets in vitro and in vivo. Based on these initial cleavage analyses, the ribozyme that was ultimately selected targeted the gene product for the HSV late gene U_L20. An adenoviral vector was then used to express this ribozyme in cell culture or in mice to assess the ability of this ribozyme to inhibit HSV-1 replication.

Although the mechanism of HSV-1 virus maturation and egress to the extracellular space is not fully understood, it has been shown that U_L20 protein, an essential gene product, plays an important role in this aspect of viral replication in cell culture (1). The HSV-1 U_L20 gene is highly conserved in alphaherpesviruses, e.g., varicella-zoster virus (4), bovine herpesvirus 1 (29), and pseudorabies virus (15), as well as in the gammaherpesvirus Marek’s disease virus type 2 (12). The U_L20 open reading frame, which is positionally conserved among alphaherpesvirus genomes, encodes a 222-amino-acid nonglycosylated membrane protein and is regulated as a γ1
gene. Deletion of the HSV-1 U20 and the pseudorabies virus U20 genes has been shown to reduce infectious virus production by up to 100-fold compared to that of wild type (1, 6, 9). In the absence of the U20 protein, virions are trapped in the perinuclear space, as well as in cytoplasmic vesicles of the host cell, and therefore, infectious virions are not released to the extracellular space. Although it has been recognized as a membrane protein, the U20 protein is also involved in Golgi apparatus-dependent glycosylation and cell surface expression of glycoprotein K; both glycoprotein K and U20 are required for syncytium formation during HSV-1 infection. Therefore, U20 is also involved in virus-induced cell fusion.

Previous attempts to inhibit HSV-1 replication by gene targeting have focused largely on inhibiting immediate early gene expression, e.g., that of ICP4 (27, 28). In this study, the impact of knocking down a late gene of HSV-1 was addressed. Using an adenoviral vector to express a hammerhead ribozyme targeting U20 RNA (Ad-U20 Rz), expression of the U20 gene was significantly reduced, leading to an inhibition of HSV-1 viral replication in vitro and in vivo. These findings suggest a possible new approach for therapy for HSV-1 infections.

MATERIALS AND METHODS

Ribozyme kinetics in vitro. We selected several genes as ribozyme targets, based on their requirements for viral replication (18). Cleavage sites for hammerhead ribozymes were selected as previously described (25), and two ribozymes were tested for each target mRNA. Synthetic RNA oligonucleotides comprising hammerhead ribozymes and corresponding target sequences were purchased from Dharmacon, Inc. (Lafayette, CO). The cleavage activity of each ribozyme was studied kinetically, as described previously (8).

Ribozyme cloning and packaging in an adenoviral vector. Ribozymes were cloned into the pAdlox shuttle plasmid (GenBank accession number R62024) for packaging into an adenoviral vector. The hammerhead ribozyme was cloned between the cytomegalovirus promoter sequence and the simian virus 40 poly(A) signal. Following cloning of the hammerhead ribozyme, we inserted a hairpin ribozyme that separates the antiviral hammerhead ribozyme from a downstream internal ribosome entry site-green fluorescent protein (IRES-GFP) element. The shuttle plasmid was then inserted into the κβ adenoviral backbone by a cis-acting replication element (CRE)-for recombination. The recombinant vectors were then propagated in creb 293 cells (11, 20). Adenoviral purification was conducted using a Vivapure AdenoPACK 100 (Vivasience AG, Hannover, Germany). The virus particle concentration and the 293 cell infectious titer of the adenovirus stock were determined as described previously (20). Two adenoviruses, Δ5, the parental vector for transgene packaging (11), and Ad-GFP, which expresses GFP from the Δ5 adenoviral backbone by a cis-acting replication element (CRE)-for recombination, were used as controls in this study.

HSV-1 preparation. A low-passage stock of HSV-1 strain 17syn+ was prepared from a master stock obtained from J. Stevens (UCLA). The drug-resistant strains of HSV-1, PAAR5 (2, 13), tkLTRZ1 (3, 14), and ACCGr4 (2), as well as their parental strain, KOS, were generously provided by D. Coen (Harvard Medical School). The HSV-1 virus was propagated and titered on rabbit skin cells grown in minimal essential medium supplemented with 5% calf serum and antibiotics (26).

Ribozyme inhibition of multiple-step HSV-1 replication. All infections were performed in triplicate. Three groups were included to test the effect of the ribozyme in a wild-type HSV-1 infection: mock treatment, Δ5 (backbone adenovirus), and Ad-U20 Rz. Each experiment was performed in triplicate. Rabbit skin cells were seeded at a density of 2 × 10^5 cells per well (3.5 cm²), followed by a drug-adenoviral inoculation at a dose of 7.5 × 10^5 virus particles per cell for 15 h. The infection of HSV-1 (17syn+) was conducted at a multiplicity of infection (MOI) of 10⁻³ for 24 h before cell lysates were harvested for titration. Titration was conducted by serial dilution of cell lysates, and PFU were counted on rabbit skin cells, in triplicate for each dilution. After the yields were calculated, average infectious yield values and standard deviations were calculated from the three separate replicates of each experiment.

Four groups were included to test the impact of the vector-expressed ribozyme on the replication of drug-resistant HSV-1 strains: mock infection and Ad-U20 Rz treatments were performed as described before; an adenovirus expressing GFP was used instead of Δ5, and an acyclovir (ACV) treatment (0.1 μM) was included. The dosage of acyclovir was determined by the threshold dose needed to distinguish drug resistance of HSV-1 (25). Forty-eight hours after infection with HSV-1, cell lysates were harvested for titration. Five HSV-1 strains were tested: the drug-sensitive strains 17syn+ and KOS and the drug-resistant isolates PAAR5, tkLTRZ1, and ACCGr4.

Ribozyme inhibition of U20 expression. We evaluated the effect of the vector-expressed U20 ribozyme on single-step growth of HSV-1 (strain 17syn+). Each experiment was conducted in triplicate, and each experiment included three treatments: mock infection, Ad-GFP, and Ad-U20 Rz. After Ad-U20 Rz was inoculated as described above, HSV-1 infection was conducted at an MOI of 3 for 8 h before cell lysates were harvested using TRizol reagent (Invitrogen, Carlsbad, CA). Total RNA and DNA were separated by following the manufacturer’s recommendation, and total RNA was treated with RNase-free DNase (10) to remove the DNA contamination. Reverse transcription was conducted using a First-Strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ), followed by real-time PCR (16). Specific primers and probes for either the viral genes (Table 1) or the rabbit GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene were synthesized by Applied Biosystems (Foster City, CA).

Testing the ribozyme inhibition of HSV-1 encephalitis in mice. Six-week-old female ND4 Swiss mice (Harlan-Sprague Dawley, Inc.) were infected via the rear footpads as previously described (16). Three groups of 10 mice were employed, and two independent experiments were conducted. First, 1.4 × 10¹⁰ virus particles of adenovirus (Δ5 or GFP) or phosphate-buffered saline (PBS) in a volume of 10 μl per footpad was combined with 40 μl of 10% sterile saline solution and injected into both rear footpads. After 3 to 4 h, mice were inoculated again with 1.4 × 10¹⁰ virus particles of adenovirus (U20 Rz or GFP) or PBS applied topically to each rear footpad following light scarification of the footpad with an emery board. This second application of adenovirus or PBS was immediately followed by a topical application of HSV-1 (17syn+) at a dose of 10¹⁰ PFU per footpad.

RESULTS

Kinetic analysis of HSV-1 ribozymes. Initially, four essential HSV-1 genes were chosen as ribozyme targets, ICP4, U20, U2030/DNA polymerase, and U54/ICP27. Two ribozyme cleavage sites were selected for each gene, based on their moderate G+C content and their predicted accessibility within the mRNA (33) and on other established design and validation parameters for therapeutic ribozymes (7). Sequences of ribozymes and their cleavage sequences are shown in Table 2. The ribozymes were evaluated for their catalytic efficiency in vitro on synthetic target RNAs, using time course and multi-turnover kinetic analysis. The time course of cleavage was...
established at a 10-fold excess of substrate relative to the amount of ribozyme and at a Mg\(^2+\) concentration of 5 mM, unless the rate of cleavage was low, in which case 20 mM Mg\(^2+\) was also employed. The U\(_{120}\) Rz154, for example, led to 75% cleavage of its RNA target by 8 min in the presence of low Mg\(^2+\) (Fig. 1). The dependence of the initial rates of cleavage on substrate concentration was then determined and analyzed using Lineweaver-Burk double-reciprocal plots to determine \(K_m\), \(k_{cat}\) (\(V_{max}/[Rz]\)), and \(k_{cat}/K_m\) values (Fig. 1C). The value for \(k_{cat}/K_m\), which is an estimate of catalytic efficiency, ranged from 0.01 for U\(_{120}\) Rz135 to 15.9 for U\(_{120}\) Rz154 (Table 3). For each HSV-1 target gene, the most efficient ribozyme was chosen for analysis as an inhibitor of viral replication.

The HSV-1 U\(_{120}\) ribozyme was the most efficient at inhibiting HSV-1 replication in vitro. Synthetic ribozymes for each of the four genes were then transfected into rabbit skin cells and tested for their abilities to reduce viral yields following infection with HSV-1, as described in Materials and Methods. These analyses demonstrated that the U\(_{120}\) ribozyme consistently resulted in the greatest reduction in viral yields (data not shown). Ribozymes targeting immediate early genes (ICP4 and UL54) did not lead to a significant inhibition in viral replication, but ICP4-Rz885 mRNA displayed low catalytic efficiency (\(k_{cat}/K_m = 0.3\)), which may explain its low inhibition of replication.

The vector-expressed ribozyme targeting the HSV-1 U\(_{120}\) mRNA significantly reduced wild-type viral replication in rabbit skin cells. Based on a screening of the above-described results, the U\(_{120}\) Rz154 ribozyme was determined to be the most efficient at inhibiting HSV-1 infection following transfection. Therefore, a DNA cassette expressing U\(_{120}\) Rz154 was cloned into an adenoviral vector in order to provide a more

### Table 2. Ribozyme sequences and sequences of their respective targets

<table>
<thead>
<tr>
<th>Ribozyme*</th>
<th>Ribozyme sequence</th>
<th>Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP4-885</td>
<td>ACGAACUGAUGACGGCUUCGGCGCGAAAGGAGAUG</td>
<td>CAUCCCCUUCUGU</td>
</tr>
<tr>
<td>ICP4-533</td>
<td>UCAUACUGAUGACGGCUUCGGCGCGAAAGCGCG</td>
<td>CGGCCGAUCAGA</td>
</tr>
<tr>
<td>U(_{120})-135</td>
<td>GAACUCAUGAUGACGGCUUCGGCGCGAAACAAAA</td>
<td>UUUUGUCAGUUC</td>
</tr>
<tr>
<td>U(_{120})-154</td>
<td>CGGAACUCUGAUGACGGCUUCGGCGCGAAACGGCA</td>
<td>UGGGCUUCUGCG</td>
</tr>
<tr>
<td>U(_{130})-933</td>
<td>AGGGUCUGAUGACGGCUUCGGCGCGAAACGGCA</td>
<td>GGUCCGACCCAUU</td>
</tr>
<tr>
<td>U(_{130})-1092</td>
<td>CACAUCUGAUGACGGCUUCGGCGCGAAACCUGAC</td>
<td>CACAGGCUACAG</td>
</tr>
<tr>
<td>U(_{154})-233</td>
<td>UUGCAUCUGAUGACGGCUUCGGCGCGAAACAGGGA</td>
<td>UCUGGCUACAGAA</td>
</tr>
<tr>
<td>U(_{154})-825</td>
<td>UGCAUCUGAUGACGGCUUCGGCGCGAAACAGGGA</td>
<td>ACAGGCUACAGGA</td>
</tr>
</tbody>
</table>

* The ribozyme name is composed of the name of the target gene (e.g., ICP4) and the cleavage site nucleotide (e.g., 885).

### Table 3. Summary of in vitro kinetic analyses of hammerhead ribozymes designed against HSV-1

<table>
<thead>
<tr>
<th>HSV target gene</th>
<th>Mg(^2+) (mM)</th>
<th>(k_{cat}) (min(^{-1}))</th>
<th>(K_m) ((\mu)M)</th>
<th>(k_{cat}/K_m) ((\mu)M(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP4–885</td>
<td>20</td>
<td>15.87</td>
<td>52.83</td>
<td>0.3</td>
</tr>
<tr>
<td>ICP4–533</td>
<td>5 and 20(^{b})</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>U(_{120})-135</td>
<td>20</td>
<td>0.08</td>
<td>5.64</td>
<td>0.01</td>
</tr>
<tr>
<td>U(_{120})-154</td>
<td>5</td>
<td>27.78</td>
<td>1.75</td>
<td>15.9</td>
</tr>
<tr>
<td>U(_{130})-933</td>
<td>20</td>
<td>9.26</td>
<td>2.57</td>
<td>3.6</td>
</tr>
<tr>
<td>U(_{130})-1092</td>
<td>5</td>
<td>22.99</td>
<td>23.59</td>
<td>1.0</td>
</tr>
<tr>
<td>U(_{154})-233</td>
<td>5</td>
<td>0.91</td>
<td>8.58</td>
<td>0.1</td>
</tr>
<tr>
<td>U(_{154})-825</td>
<td>5</td>
<td>51.28</td>
<td>4.44</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* NA, no activity detected.

* No activity was detected at either Mg\(^2+\) concentration.
efficient means of delivering this ribozyme for subsequent analyses. This adenoviral vector (Ad-U_{120} Rz154) was then tested for its ability to inhibit HSV-1 replication in rabbit skin cells. HSV-1 infection was conducted at a low MOI (10^{-3}), and the ribozyme was used to inhibit multistep HSV-1 viral replication. Ad-U_{120} Rz154 reduced HSV-1 wild-type viral replication by 83% compared with that of the adenovirus vector (without ribozyme) control treatment (P < 0.001), and no significant differences were observed between the groups that received no treatment and those that received Ad-GFP treatments (Fig. 2).

Ad-U_{120} treatment blocked the U_{120} gene expression during HSV-1 infection of rabbit skin cells. Ad-U_{120} Rz154 was also tested with a one-step replication assay of HSV-1 following infection at an MOI of 3. As U_{120} is a late gene, an 8-h incubation was used to allow completion of late gene expression. The effect of U_{120} Rz154 on U_{120} expression at the mRNA level was assayed by reverse transcription, followed by real-time PCR. A 68% reduction in U_{120} mRNA level was detected by comparing results of the Ad-ribozyme treatment with that of the Ad-GFP treatment (P < 0.0005) (Fig. 3B). There was also a 50% reduction in U_{120} mRNA level using Ad-GFP treatment relative to that of mock infection (P < 0.0004). It is therefore likely that preinfection of the cells with adenoviral vectors reduces the replication of HSV-1. DNA was also extracted from each infected sample, and the viral DNA level normalized to that of the endogenous cellular control (GAPDH) was also determined using real-time PCR (Fig. 3A). A 54% reduction in viral DNA level was observed for the ribozyme treatment group compared to that of the Ad-GFP group (P < 0.0004), but there were no significant differences between the DNA levels of the mock infection and those of the Ad-GFP treatments.

Ad-U_{120} Rz154 inhibits viral replication of HSV-1 drug-resistant strains. Treatment with Ad-U_{120} Rz154 was compared with that with acyclovir, using multistep infection at an MOI of 10^{-3}. Ad-U_{120} Rz154 significantly inhibited wild-type HSV-1 viral production (by 98% against 17syn+ and by 95% against KOS), and acyclovir treatment led to similar levels of inhibition (99% and 80%, respectively). However, acyclovir treatment was not effective against drug-resistant strains of HSV-1 (no significant differences in HSV yields), while the U_{120} ribozyme maintained its inhibitory effect. In the case of PAAr5 infection, the U_{120} ribozyme reduced viral production by 99%; reductions were 76% for tkLTRZ1 infection and 70% for ACGr4 infection (Fig. 4). Therefore, knocking down the expression of the U_{120} gene led to a significant inhibition of HSV-1 viral replication in viral strains that were resistant to a conventional antiviral drug. It should be noted that the 70- to 100-fold reduction of viral yields shown in Fig. 4A and B are greater than those shown in Fig. 2 because the infectious virus was harvested at 48 h in the experiments shown in Fig. 4 as opposed to 24 h as shown in Fig. 2.

The vector-expressed U_{120} ribozyme protects mice following footpad infection with HSV-1. To evaluate the impact of blocking the expression of the U_{120} gene in vivo, Ad-U_{120} Rz154 was delivered to the mouse footpad prior to infection with HSV-1. Viral particles (1.4 × 10^{10}) of either Ad-U_{120} Rz154 or Ad-GFP were injected into both rear footpads. Three hours later, mice were challenged with HSV-1 (10^{4} PFU of 17syn+ per footpad). Beginning at day 6 postinfection, mice from the
control groups (receiving Ad-GFP and PBS treatments) showed signs of encephalitis, including hind-limb paralysis, hunched posture, ruffled fur, ataxia, and weakness. Mice that eventually became nonresponsive and could not ambulate were euthanized. However, mice from the ribozyme treatment group remained healthy and active overall (though at a much later time point, two deaths were observed, and one mouse showed mild paralysis). With the HSV ribozyme treatment, 89% of animals survived, while 45% and 40% survival rates were observed for PBS- and Ad-GFP-treated controls, respectively (Fig. 5). Kruskal-Wallis nonparametric analysis showed significant differences among the three groups ($P = 0.04$).

To further investigate the ribozyme’s impact on viral replication in vivo, an independent experiment was performed in which four or five mice per group (receiving the Ad-ribozyme or Ad-GFP control treatment, respectively) were sacrificed at 6 days postinfection, and the feet, dorsal root ganglia, and spinal cords were dissected. Real-time PCR was conducted to compare viral DNA levels from different tissues. While Ad-ribozyme treatment resulted in decreased HSV-1 viral DNA recovery from all tissues, the decrease did not reach statistical significance. In the spinal cord assay, where day 6 represents the peak of viral DNA loads, Ad-ribozyme treatment reduced the amount of viral DNA by sixfold (Fig. 6).

**DISCUSSION**

In this study, hammerhead ribozymes were designed to limit the expression of several HSV-1 essential genes (ICP4, U1_54, U1_30, and U1_20). In vitro kinetic analysis led to the selection of ribozymes with the greatest potential for efficient target cleavage. Because the U1_20 Rz154 exhibited the most robust inhibition of viral replication following transfection, it was cloned into an Ad5 vector and tested further in vitro and in vivo.

Our best target and best ribozyme was the U1_20 gene, a γ1 gene, encoding a membrane protein that is essential for viral intra- and extracellular egress, as well as intracellular transport of viral glycoproteins. The ribozyme targeting U1_20 mRNA, which also likely disrupts U1_20.5 mRNA (32), not only reduced the replication of wild-type HSV-1 (in strains 17syn+ and KOS) but also inhibited the replication of drug-resistant HSV-1 strains (PAAr5, tkLTRZ1, and ACGr4). Data presented here confirm that the U1_20 gene product of HSV-1 is

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**FIG. 4.** Ad-U1_20 Rz is effective in inhibiting acyclovir-resistant strains of HSV-1. After treatments, cells were infected with the indicated HSV strain at an MOI of $10^{-3}$ for 48 h. (A) Ad-ribozyme treatment led to a significant reduction (by 98%) in 17syn+ viral replication compared with Ad-GFP treatment ($P < 0.002$), and acyclovir treatment had very similar inhibitory effect (99% reduction; $P < 0.02$). (B) Ad-ribozyme inhibited the viral replication of HSV-1 strain KOS by 95% compared with Ad-GFP treatment ($P < 0.05$), while ACV inhibited KOS by 80% ($P < 0.02$). (C) The HSV-1 drug-resistant strain PAAr5 could be inhibited by ribozyme (99% reduction; $P < 0.005$) but not by ACV. (D) The drug-resistant strain tkLTRZ1 viral replication was inhibited by ribozyme by 76% ($P < 0.05$), while there was no effect from ACV. (E) The double-mutant strain ACGr4 viral replication was inhibited by ribozyme by 70% ($P < 0.006$), while ACV had no effect.
essential for the virus life cycle in rabbit skin cells and that decreasing the expression of this gene leads to a reduction of viral production. Since UL20 is a late gene involved in virion maturation, our results indicate that these steps, including viral egress, may be suitable targets for antiviral intervention.

A control adenovirus, Ad-GFP, was included to assess vector effects. When UL20 mRNA levels were evaluated for the ribozyme effect, Ad-GFP treatment led to an approximately 50% reduction (Fig. 3B), and a similar reduction was observed at the level of mRNA for the viral DNA polymerase (data not shown). The Ad vectors we employed contained deletions in the E1 and E3 regions but still contained viral late genes. It is possible that Ad-GFP competed with HSV-1 for the usage of cellular machinery, e.g., RNA polymerase II, indirectly leading to a lower level of viral gene expression in the Ad-GFP treatment group. Overall, in spite of the nonspecific effect of adenovirus, UL20 Rz154 reduced viral target gene expression and inhibited virus replication significantly compared with the Ad-GFP treatment.

An interesting finding from this study was the significant reduction in viral DNA yields observed when the UL20 ribozyme was tested with a single-step infection assay (Fig. 3A). It has been previously shown that UL20 expression is diminished when HSV-1 DNA replication is blocked, consistent with the designation of UL20 as a late gene (31). It was therefore surprising that the inhibition of this late gene by the ribozyme following a high MOI resulted in a decrease in viral DNA accumulation. These results suggest that UL20 expression provides a feedback signal to viral DNA synthesis.

The Ad-UL20 Rz154 ribozyme was capable of inhibiting the replication of acyclovir-resistant strains of HSV-1 (PAAr5, tkLTRZ1, and ACGr4) (Fig. 4), which were chosen because they represent two general drug resistance mechanisms: mutations in thymidine kinase and in DNA polymerase. Most of the antiviral drugs currently approved for treatment of HSV-1 infection are nucleotide analogs, which are either substrates of thymidine kinase that indirectly disrupt viral DNA synthesis or are incorporated into the elongated DNA strand and lead to premature termination. The treatment of patients, especially those with immune deficiency and repeated reactivations, with antivirals has led to the selection of drug-resistant viruses that results in the uncontrolled spread of HSV-1 infection. In some cases, these infections are lethal to patients. It is encouraging that a nucleic acid-based approach such as a ribozyme targeting
mRNA of HSV-1 U120 might overcome this resistance. Although nucleotide changes can cause the emergence of resistant escape mutants for the U120 ribozyme, ribozymes targeting different essential genes of HSV-1 can be combined to guarantee their inhibition, e.g., by combining ribozyme-targeting immediate early genes and early and late essential genes. The use of a ribozyme-based therapy may be particularly applicable to HSV infections of the cornea, where the toxicity of the currently available antivirals poses a problem.

The mouse footpad infection model offers an efficient approach with which to study HSV-1 viral neuroinvasion, neurovirulence, and latency. We applied Ad-U120 Rz154 to block the initial replication in footpad epithelium, thereby reducing the retrograde transport of HSV-1 to the dorsal root ganglion (24). At an inoculum of 10^6 PFU of wild-type HSV-1, the viral replication in the footpad epithelium led to severe damage in the central nervous system, which caused death in 60% of our control animals. Pretreating the mouse footpad with Ad-ribozyme consistently led to a significant level of protection (an overall 89% survival rate) against the HSV-1 challenge. Despite the HSV challenge, most animals in this group remained healthy throughout the study; one mouse showed mild paralysis of the hind limbs but remained active after the study’s end point. In contrast, in both of the control groups, death and the indication of severe damage in the central nervous system were observed.

Interestingly, the ribozyme targeting a late essential gene, U120, achieved the most significant therapeutic effect against HSV-1 infection in vitro and in vivo, in this study. Palliser et al. designed siRNAs against HSV-2 U27 and U29, which encode an envelope glycoprotein and a DNA binding protein, respectively (19). Their results indicate that suppressing the expression of early/late genes of HSV has a greater impact on the virus lytic life cycle than inhibiting the expression of immediate early genes. Our results here demonstrated that targeting a late gene was more effective than targeting genes of the other kinetic classes. We hypothesize that significantly inhibiting viral protein production of genes encoding structural proteins or virion maturation might produce a more profound effect than inhibiting regulatory genes, whose products are required in smaller and nonstiostochiometric amounts. We have extended this finding to demonstrate that a nucleic acid-based therapeutic agent (U120 Rz-154) targeting a late gene could inhibit the viral replication of drug-resistant HSV-1 strains and could limit the lethal effects of an HSV-1 infection in vivo. Our next step is to develop a delivery system that will permit long-lasting expression of such therapeutics in the corneal epithelium.

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