Effect of Deletion of the Ribonucleotide Reductase Gene in Wild Type and Virion Associated Host Shutoff (vhs-1) Mutant Herpes Simplex Virus-1 on Viral Proliferation and Infected-Carcinoma Cell Cultures Growth

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Abstract:

Glioblastoma multiforme is the most prevalent and deadliest form of glioma and brain cancer, with a very poor prognosis. In an effort to develop an oncolytic viral vector for the treatment of Glioblastoma multiforme, we replaced the UL39 and UL40 genes encoding ribonucleotide reductase (RR) with green fluorescence protein and luciferase genes in wild type KOS and in the virion host shutoff mutant vhs-1, resulting in strains KOS-RR and Vhs-RR, respectively. KOS-RR and Vhs-RR caused death of infected U87 Glioblastoma multiforme cell cultures within one day after infection, whereas KOS and vhs-1-infected cells were more viable. All four viral strains caused apoptotic DNA laddering in infected H1299 lung cancer cells, while only Vhs-RR caused apoptosis in U87 cell cultures. Vhs-RR gave higher yields on U87 than on Vero cells, while it barely proliferated on non-dividing Goiter cells. These results indicate that Vhs-RR proliferates well in actively growing U87 Glioblastoma multiforme cells, causing their death in a mechanism involving apoptosis, while sparing non-dividing cells. Therefore, Vhs-RR is a promising candidate for oncolytic treatment of brain tumor malignancies.

Keywords: herpes simplex virus - 1, virion associated host shutoff mutant, ribonucleotide reductase, oncolytic viral therapy, glioblastoma multiforme.

Introduction

Malignant gliomas are the most common primary brain tumors in all ages, arising from glia or their precursors in the central nerve system (CNS). Glioblastoma multiforme (GBM) is the most prevalent and deadliest form of glioma and brain cancer. Despite intense efforts in the past years, GBM is still not responsive to conventional surgical, radio-therapeutic and/or chemo-therapeutic interventions [32], mainly because of its aggressive, invasive and destructive malignancy nature, together with its high proliferation rate [39]. As a result, patients with GBM have a poor prognosis, with a median survival of 12–14 months, with population-based studies indicating even shorter median survival [50]. Only 3–5% of GBM patients survive for more than 3 years [34]. However, because of its nature, GBM is an excellent target for novel molecular approaches for its treatment. GBM cells are among the few rapidly proliferating and actively dividing cells in the CNS, and the tumor's fatality is mainly caused by local growth and local recurrence, thus therapeutic activity is only needed locally and in dividing cells. The novel molecular approaches for the treatment of GBM that have been investigated include immunotherapy, gene therapy, oncolytic virotherapy and multimodal molecular therapy that combines more than one approach. Some of these approaches have demonstrated promise in preclinical and early clinical studies [60]. The vectors proposed for oncolytic virotherapy include those derived from retroviruses, adenoviruses, adeno-associated viruses, reoviruses and herpesviruses [3].

Herpes simplex virus 1 (HSV-1) is one of the successful agents of oncolytic virotherapy currently undergoing clinical trials. It is an enveloped, double-stranded DNA virus with a genome size of 152 kb. Several features of this virus make it attractive for gene therapy: it can be made in high titers and infect a wide range of dividing and non-dividing cells, including tumor cells of human and rodent origins; as much as 30 kb of its the genome may be replaced by foreign genes in replication-defective HSV-1 mutants; HSV-1 rarely produces severe medical illness in immune-competent adults; antiherpetic agents such as acyclovir are available that provide a

safety mechanism to shut off viral replication should systemic toxicity ensue; HSV-1 does not integrate its genome into the cellular genome but rather remains as an episome within the infected cell, so insertional mutagenesis is not a concern; and it is cytolytic by nature [3, 40, 68].

One approach that was successfully demonstrated with HSV-1 is using it as a conditionally replicating virus, in other words – a virus that is capable to replicate primarily in actively growing cells, i.e., cancer cells, thus causing lysis mainly of the infected cancer cells. One way to generate a replication-conditional mutant of HSV-1 was to delete from the viral genome the ribonucleotide reductase (RR) genes [3]. RR is a key enzyme in DNA biosynthesis of all eukaryotic and prokaryotic organisms. It reduces ribonucleotides to their corresponding deoxyribonucleotides, thus providing a major pathway in the synthesis of DNA precursors [48]. HSV-1 encodes its own RR, which is composed of two non-identical subunits having molecular weights of 140-kDa and 38-kDa [11]. They are encoded by adjacent genes - UL-39 and UL-40, respectively, which map to the U_L region of the HSV-1 genome [56]. The cellular RR enzyme is not available in non-dividing cells, like neurons, whereas it is largely active in rapidly replicating cancer cells. Since viral replication can only take place in the presence of RR, viruses deleted of this enzyme activity can replicate only in actively dividing cells [59]. The *Escherichia coli* lacZ gene was inserted into the viral RR gene locus in frame with the amino terminal portion of RR. This insertion deleted RR expression and added lacZ expression to the resulting HSV-1 strain – hrR3. The growth of this mutant strain was severely compromised in serum-starved cells compared to exponentially growing Vero cells [22, 23]. Further studies with hrR3 have shown that compared to wild-type HSV-1 its virulence in eye infection in mouse model systems is greatly reduced compared to wild-type HSV-1 due to its reduced ability to grow in the infected organ [13, 14]. hrR3 was found also hypersensitive to the antiherpetic agent ganciclovir, thus rendering it safer to be used as an oncolytic viral agent than wild-type HSV-1 [42]. Infection with hrR3 successfully destroyed cultured U87 MG human GBM cells [42], colon carcinoma cell lines [72] and cultured PANC-1 human pancreatic carcinoma cells [63]. In vivo studies have shown that treatment of mice transplanted with U87 MG tumors with hrR3 significantly inhibited tumor growth, whereas the expression of LacZ fused to RR large subunit allowed the detection of the mutant virus in the treated tumors, using 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) histochemistry [42]. This mutant was used successfully for the treatment of other cancer model systems in mice, like human colon cancer [70] and human ovarian cancer [46]. Similar results were obtained with other mutant strains of HSV-1 lacking RR activity, for instance the temperature-sensitive mutant in the RR small subunit - ts1222. In the permissive temperature, ts1222 grew like the wild-type virus, whereas at the non-permissive temperature the growth of ts1222 was severely impaired in serum-starved non-growing cells [55]. Another mutant of HSV-1, in which most of the RR large subunit was deleted, was extremely impaired in its ability to replicate acutely in the eye of infected mice and in the trigeminal ganglion [28], as well as in the vagina, or to cause death in mice following intracerebral, intraperitoneal, or intravaginal inoculation, or in guinea pigs following intraperitoneal or intravaginal inoculation [27]. RR deletion mutants also failed to replicate in brains of mice greater-than-or-equal-to 8 days old but exhibited significant virulence for newborn mice as a result of viral replication in the brains [69].

Other mutant strains carrying additional mutations except the inactivation of RR were also used successfully as oncolytic vectors for the treatment of cancer. G207 is a double mutated HSV-1 strain. It has deletions at both γ 34.5 loci and a lacZ gene

insertion inactivating UL39. This mutant has the ability to replicate in glioblastoma cells, attenuated neurovirulence, temperature sensitivity, ganciclovir hypersensitivity, and the presence of an easily detectable marker. G207 kills human glioma cells in monolayer cultures, and decreases tumor growth and/or prolongs survival of mice carrying U87 MG tumor xenografts. It is also avirulent upon intracerebral inoculation of mice and HSV-sensitive non-human primates [43]. G207 passed successfully a dose-escalation phase I clinical trial and has undergone further clinical testing [60].

Another HSV-1 mutant that has the potential to be used as an oncolytic vector is vhs-1. The HSV-1 virion host shutoff (vhs) protein is encoded by UL41. Upon infection, copies of the vhs protein enter the cell as components of the virion tegument and shut off host protein synthesis by mediating the degradation of preexisting and newly transcribed mRNAs during the first few hours after infection [12, 20, 35, 58, 61, 64, 65, 66]. This protein was found to harbor an endoribonucleolytic activity [66], with substrate specificity similar to that of RNase A [65]. Because the vhs protein comes in the infecting particle the shutoff begins immediately, prior de-novo viral gene expression [21, 36, 57, 64]. The vhs mRNAse targets the bulk of infected cell mRNAs, including house-keeping genes, actin, tubulin, stress induced genes, e.g., the immediate early stress response gene IEX-1, c-fos, I kappa B alpha, heat shock 70 mRNA, and mRNAs of antiviral host immune response [38, 51, 61, 64]. A HSV-1 mutant defective in the virion-associated shutoff of host polypeptide synthesis, termed Vhs-1, was isolated [57]. It has a threonine to isoleucine transition in amino acid 214 of the protein, which inactivates the mRNAse activity [35, 36]. In contrast to wild-type virions, this mutant did not cause shutoff of host protein synthesis and was found to be defective in the ability to degrade host mRNA. Infection of primary cultures of mouse cerebellar granule neurons (CGNs) with Vhs-1 induced apoptotic cell death at earlier times than wild-type virus. In addition, wild-type HSV-1 replicated well in the CGNs, whereas there was no apparent replication of the Vhs-1 mutant virus [12]. HSV-1 mutants lacking vhs protein were found to be severely attenuated in animal models of pathogenesis and exhibited reduced growth in primary cell culture. This is attributed to the accumulation of viral RNAs in the vhs-depleted virus-infected cells, which induce expression of antiviral genes in a higher extent than in wild-type HSV-1-infected cells [51]. Based on these findings, using the Vhs-1 mutant as a vector for treatment of brain cancer seems to be safer than using the wild-type virus, because its reduced apparent replication in non- or poorly-dividing cells.

Using single mutant HSV-1 vectors poses risks including recombination with latent host virus to restore wild-type phenotype, reactivation of latent virus in the host and suppressor mutations to restore wild-type phenotype. One solution to these risks is to generate vectors with dual mutations [3]. RR-deleted HSV-1 was investigated in the past, as is or in combination with other mutations, as a potential agent for cancer therapy. The current research is the first attempt to investigate the effect of a combination of RR deletion and the inability to shutoff host protein synthesis (vhs) on infected human U87 MG (GBM) and H1299 (lung cancer) cells.

Materials and Methods

Cell lines

Vero (African green monkey kidney) and H1299 lung carcinoma cells were obtained from Prof. Bernard Roizman, The University of Chicago (Chicago, IL, USA). The permanent human GBM cell line U87 MG was provided by Prof. M. Oren from the

Weizmann Institute of Science (Rehovot, Israel). Primary Goiter cells were provided by Prof. Zachi Chrain (The Technion, Haifa, Israel), Meital Cohen and Dr. Gary Weisinger (Tel Aviv Sourasky Medical Center, Tel Aviv, Israel). Vero cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal calf serum and 4 mM L-glutamine. U87 MG cells were maintained in DMEM supplemented with 10% inactivated fetal calf serum, 1% MEM-EAGLE with Non-Essential Amino Acids, 2 mM L-glutamine, 1 mM Sodium Pyruvate, 100 units/ml Penicillin and 100 µg/ml Streptomycin. H1299 cells were maintained in RPMI-1640 with 10% inactivated fetal calf serum, 100 units/ml Penicillin and 100 µg/ml Streptomycin. Primary Goiter cells were cultivated in RPMI-1640 with 10% inactivated fetal calf serum, 1% MEM-EAGLE with Non-Essential Amino Acids, 2 mM L-glutamine, 1 mM Sodium Pyruvate, 100 units/ml Penicillin and 100 µg/ml Streptomycin. All culture media components were obtained from Biological Industries Beit Haemek (Beit Haemek, Israel). All cell lines were maintained at 37°C and 5% CO₂.

Viruses

Wild type HSV-1 strain KOS was originally isolated by K.O. Smith, Baylor University, Houston, Texas. The virion associated host shutoff (vhs-1) mutant was derived in N. Frenkel's laboratory [57]. All viral strains were grown and tittered on Vero cells, and maintained in 199v - M-199 (Earle's salts base with L-glutamine) supplemented with 1% inactivated fetal calf serum) as described previously [19, 44].

Molecular biological techniques

Viral DNA was purified from virus-infected Vero cell cultures using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Plasmid mini-preparation was performed using the HiYield Plasmid Mini Kit (Real Biotech Co., Taipei, Taiwan). Plasmid maxi-preparation was performed using the Plasmid DNA Purification Kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany). DNA fragments were purified from agarose gels using the HiYield Gel/PCR DNA Extraction Kit (Real Biotech Co.). Restriction and DNA modifying enzymes were all obtained from New England Biolabs (Beverly, MA, USA). Polymerase chain reaction (PCR) was performed with 2X Ready Mix for PCR (Bio-Lab, Ltd., Jerusalem, Israel). Each reaction contained 20 pmol of each primer (Integrated DNA Technologies, Skokie, IL, USA) and 250 ng viral DNA or 100 ng plasmid DNA as templates, in a total volume of 50 µl. Thermo-cycling was performed on Eppendorf MasterCycler Gradient and consisted of pre-denaturation at 94°C for 2 min, 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, and additional elongation at 72°C for 10 min.

Construction of the RR Deletion Plasmid

In the RR deletion plasmid, the reporter genes EGFP and hRluc were cloned between sequences that flank the UL39 and UL40 genes in the HSV-1 genome, termed H1 and H2 (Homologous sequence 1 and 2). H1 is a 1 kb fragment located upstream to UL39 and H2 is a 1 kb fragment located downstream to UL40. Homologous recombination between the H1 and H2 in the RR deletion plasmid and the corresponding sequences in the HSV-1 genome should induce insertion of the EGFP and hRluc genes into the HSV-1 genome instead UL39 and UL40.

H1 and H2 were amplified with primers H1-For/H1-Rev and H2-For/H2-Rev respectively, using purified DNA of KOS-infected Vero cells as template. The coding sequence of EGFP flanked by the human cytomegalovirus (CMV) early promoter at its 5'-end and the SV40 early mRNA polyadenylation signal at its 3'-end was amplified with primers EGFP-For/EGFP-Rev using plasmid pEGFP-C1 (BD Biosciences Clontech) as template. The coding sequence of the humanized *Gaussia princeps* luciferase gene (hLuc) flanked by the human cytomegalovirus (CMV) early promoter at its 5'-end and an mRNA polyadenylation signal at its 3'-end was amplified with primers hLuc-For/hLuc-Rev using plasmid NL-GAU-LUC-H (Lux Biotechnology) as template. Each resulting PCR product was directly cloned in the PCR cloning vector pGEM-T (Promega) and its sequence was confirmed (The Sequencing Unit at The Faculty of Life Sciences, Tel Aviv University, Israel).

pGEM-T+EGFP and pGEM-T+hLuc were tested for functionality by transient transfection into Vero cells using GeneJammer Transfection Reagent (Stratagene, La Jolla, CA, USA) as described by the manufacturer and by checking fluorescence and luciferase activity of the transfected cells (results not shown).

The cloning process continued with digestion of the EGFP gene with Cla I and Asi SI and its ligation downstream to H1 in plasmid pGEM-T+H1 which was digested with the same restriction enzymes. The resulting plasmid (pGEM-T+H1+EGFP) was digested with Asi SI and Sbf I and was ligated with both hLuc that was digested with Asi SI and Avr II and H2 that was digested with Avr II and Sbf I. The resulting plasmid - pNF-1282, is shown in Figure 1. It contains EGFP and hLuc genes in opposite directions relative to each other, flanked by H1 and H2.

Homologous recombination between the RR-deletion construct and HSV-1 genome

The RR deletion construct was digested with Bln I and Sbf I. The resulting 5.38 kb fragment, which consisted of H1-EGFP-hLuc-H2 was isolated. This fragment was transiently transfected into Vero cells using GeneJammer Transfection Reagent. One day later successful transfection was confirmed by checking fluorescence under the fluorescence microscope (results not shown). Then, the transfected cells were infected with either KOS or Vhs-1, at multiplicity of infection (m.o.i.) 3, and 2 days later plaques were isolated and purified in three rounds of plaque purification using low-melting agarose on 6-well plates.

Fluorescence microscopy

Fluorescence of the virus-infected cell cultures was observed and photographed using a Nikon Eclipse TE2000-S fluorescence microscope and a Nikon digital camera DXM1200F (Nikon Instruments, Inc., Melville, NY, USA).

Cell viability assay

Cell viability of U87 and H1299 cell cultures following infection with either KOS, Vhs-1, KOS-RR or Vhs-RR was measured in 96-well plates. Each well was seeded with 2×10^3 U87 or H1299 cells, in 100 μ l medium. One day later the cells were infected with the viral strains at m.o.i. 5 or mock-infected. Cell viability was measured in the following days by adding 10 μ l (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biotium, Inc., Hayward, CA, USA) to each well, incubation at 37°C for 4 hr to allow formation of formazan crystals, addition of 200 μ l

dimethyl sulfoxide to solubilize the formazan crystals and measurement of the absorbance at 570 nm, from which the absorbance at 630 nm was subtracted. The absorbance values are directly proportional to the number of viable cells in the cultures, as indicated by the manufacturer. The results are an average of triplicates.

Apoptosis

Effect of viral infection on apoptosis was assayed by monitoring apoptotic DNA laddering. U87 and H1299 cells were seeded in 6-well plates – 2×10^5 cells per well. One day later the cells were infected with either KOS, vhs-1, KOS-RR or Vhs-RR at m.o.i. 10, or mock-infected. One, 2 and 3 days post-infection the infected cells were harvested and DNA was extracted with the SuicideTrack DNA Isolation Kit (Calbiochem) according the manufacturer's instructions. DNA fragments were separated by electrophoresis in 1.5% agarose gel stained with Ethidium Bromide.

Infectious virus yield on Vero, H1299 and U87 cell lines

2.5×10^5 Vero or H1299, or 1.7×10^5 U87 cells were inoculated per well in 24-well plates. One day later, the cells were infected with either KOS, Vhs-1, KOS-RR or Vhs-RR at 5 PFU/cell, in duplicates. The progeny viruses were harvested 24, 48 and 72 hr post-infection and their titers were determined.

Infectious virus yield on Vero and Goiter cells

Each well in a 24-well plate was inoculated with either 7.6×10^4 Vero cells or 2×10^5 Goiter cells. When the cell cultures became confluent – Vero cells three days post-infection and Goiter cells five days post-infection, they were either mock-infected or infected with KOS, KOS-RR, Vhs-1 or Vhs-RR at m.o.i. 3. During the next four days the cultures were monitored under the microscope, and viral particles were collected for determination of the infectious viral yields.

Results

Deletion of the RR gene

The plasmid that was used for the deletion of RR in HSV-1 KOS and Vhs-1 strains - pNF-1282, was constructed as described in "Material and Methods". It contains the EGFP and hLuc genes in opposite directions relative to each other, flanked by H1 and H2, which flank UL39-UL40 in the 5'- and 3'-ends, respectively (Figure 1). In order to delete UL39 and UL40 from the genome of KOS and Vhs-1, the 5.38 kb segment H1-EGFP-hLuc-H2 was digested from pNF-1282 with Bsp I and Sbf I. Then it was transfected into Vero cells, followed by super-infection with either KOS or Vhs-1 and plaque purification as described in "Materials and Methods". Following homologous recombination between the RR-deletion construct and KOS or Vhs-1 genome it is expected that EGFP and hLuc will be inserted instead of UL39 and UL40. This should cause a deletion of 4.40 kb (UL39 and UL40) and insertion of 3.44 kb (EGFP and hLuc), resulting in a net reduction of 0.96 kb in viral genome size. This should ensure proper packaging of the mutated genomes. The resulting viral strains are expected to be defective in ribonucleotide reductase activity in one hand and able to confer green fluorescence and luciferase activity to infected cells on the other hand.

PCR analysis of the recombinant viruses was performed as follows. The sequences of the primers are given in Table 1 and their locations in the viral genomes are shown in Figure 2. Primers H1-5'-For/EGFP-5'-Rev were used to check the presence of EGFP and primers hLuc-3'-For/H2-3'-Rev were used to check the presence of hLuc. As can be seen in Figure 3 the expected PCR products were obtained only in the presence of Vhs-RR. No PCR product of EGFP and hLuc was obtained with KOS, Vhs-1 and the KOS-RR strain that is shown in Figure 3. The presence of UL39 and UL40 was checked using primers H1-5'-For/UL39-Rev and UL40-For/H2-3'-Rev respectively. The expected PCR products were obtained only in the presence of KOS and Vhs-1 DNAs. No PCR product was obtained with genomes of both KOS-RR and Vhs-RR viral strains (Figure 4). These results indicate that UL39 and UL40 were successfully deleted in the KOS-RR and Vhs-RR, however they were replaced properly by EGFP and hLuc genes only in the three Vhs-RR isolates. Later on, other KOS-RR isolates were analyzed by PCR and gave the expected results (not shown).

Table 1: List of oligonucleotides.

Oligo	Sequence
H1-For (PS-4)	5'-CGCGGATCCGCTTAGCGCGGCGTTTCTGTACCTGG-3'
H1-Rev (PS-5)	5'-CGCCTGCAGCCTGCAGGCCTAGGGCGATCGCATCGATTTCACAGACGCGGCGGG-3'
H2-For (PS-10)	5'-CGCCCTAGGGCTTCTACCCGTGTTTGCCC-3'
H2-Rev (PS-11)	5'-CGC CCTGCAGGTATTAGCGCCTGCTACATTCCC-3'
EGFP-For (PS-6)	5'-CGCATCGATAGTAATCAATTACGGGGTCATTAGTTC-3'
EGFP-Rev (PS-7)	5'-CGCGCGATCGCGCAGTGAAAAAAATGCTTTATTTGTGAAATTTG-3'
hLuc-For (PS-8)	5' -CGCGCGATCGCCCCCGATTTAGAGCTTGACGG-3'
hLuc-Rev (PS-9)	5'-CGCCCTAGGACCCCAGATATACGCGTTGAC-3'
H1-5'-For (PS-18)	5'-CGCCATGGTTCACACGCAC-3'
EGFP-5'-Rev (PS-20)	5'-GGCGTTACTATGGGAACATACG-3'
H2-3'-Rev (PS-19)	5'-CTCTATCACACCAACACGGTC-3'
hLuc-3'-For (PS-21)	5'-GGAACATACGTCATTATTGACGTC-3'
UL39-Rev (PS-16)	5'-CAAAGTTGTTATCGCTGATGCGG-3'
UL40-For (PS-17)	5'-TCACCTGCCAGTCAAACGACC-3'

Viability of U87 cells following infection with wild type and mutant HSV-1 strains

The aim of this experiment was to determine the effect of infection with the RR-deletion mutants and their parental HSV-1 strains on the growth of U87 cell cultures, using MTT as an indicator for culture growth. The test results (A_{570} - A_{630}) represent the number of viable cells in the culture. As can be seen in figure 5, mock-infected cells grew continuously through the entire experiment period. U87 cells infected with KOS

and Vhs-1 were less viable, whereas cells infected with KOS-RR and Vhs-RR were barely alive throughout the experiment period.

Apoptosis

U87 and H1299 cells were infected with wild type and mutant viral strains, and apoptotic DNA degradation in the infected cells was monitored during three days post-infection. As can be seen in figures 6-9 apoptotic DNA degradation in H1299 occurred upon infection with KOS, Vhs-1, KOS-RR and Vhs-RR, whereas non-infected cells did not undergo apoptosis. On the other hand, only Vhs-RR induced apoptosis in U87 cells.

Proliferation of KOS-RR and Vhs-RR in cancer cells

Vero and U87 cells were infected with KOS, Vhs-1, KOS-RR and Vhs-RR at a ratio of 5 PFU/Cell. The infectious virus yields of the four viral strains were monitored during the following three days, and the results are shown in Figures 10 and 11.

The results given in Table 2 show the infectious virus yields of the four viral strains on Vero and U87 cells, three days post infection. The relative ratios to KOS for each cell line are also given. On Vero cells, KOS showed the highest yield, followed by Vhs-1 and KOS-RR which gave similar yields, and then Vhs-RR which gave the lowest yield on Vero. On U87, KOS-RR showed a yield slightly higher than that of KOS, followed by Vhs-RR and then Vhs-1 which showed the lowest yield on U87. When analyzing the yield of each virus strain on U87 relative to Vero, it can be seen that there is a decrease in the yields KOS and Vhs-1 on U87 compared to Vero, whereas KOS-RR showed similar yields on both cell lines, and the yield of Vhs-RR on U87 was more than two times higher than the yield on Vero.

The growth pattern of the four viral strains on Vero cells which was shown in their infectious virus yields was also reflected in the plaque sizes of these viral strains on Vero cells, as can be seen in Figure 12. The plaques formed by KOS were the biggest ones. Vhs-1 and KOS-RR caused the formation of plaques more or less similar in size, but smaller than then ones of KOS, whereas Vhs-RR gave rise to the smallest plaques on Vero cells.

Table 2: Infectious virus yield of wild type and mutant HSV-1 strains on Vero and U87 cells, 3 days post infection.

Virus	Vero (PFU/Cell)	U87 (PFU/Cell)	U87/Vero
KOS	340 (1.00)	98 (1.00)	0.29
Vhs-1	86 (0.25)	44 (0.45)	0.51
KOS-RR	120 (0.35)	114 (1.16)	0.95
Vhs-RR 1G5	32 (0.09)	71 (0.73)	2.22

The starting infection ratio was 5 PFU/cell. The numbers in brackets indicate the yields relative to KOS for each cell line. The ratios of the yields on U87 relative to Vero are also given.

Proliferation of KOS-RR and Vhs-RR in non-dividing Goiter cells

When cells isolated from Thyroid Goiter tissues are cultured *in vitro* they tend to stop growing when they become confluent, a phenomenon known as contact inhibition. Confluent Goiter cells can therefore serve as a model system for a non-growing cell culture or tissue. Goiter and Vero cells were infected with KOS, Vhs-1, KOS-RR and Vhs-RR and their infectious virus yields were monitored during a period of seven days after infection. As can be seen in figure 13 and 14, all four viral strains grew better on Vero cells, and barely grew on Goiter cells, except KOS that was the only virus that proliferated relatively well on Goiter cells.

Discussion

Deletion of RR

In the current study the two genes that encode for the RR large and small subunits - UL39 and UL40, were deleted from the genomes of a wild type strain of HSV-1 and the virion host shutoff mutant vhs-1. The deletion of RR was accompanied with the simultaneous insertion of two reporter genes – EGFP and hLuc, resulting in net reduction of 0.96 kb in the viral genome length. This should not pose any difficulties in packaging the recombinant genomes in the virion particles, which can accommodate up to ca. 150 kb DNA molecules [62].

The presence of the reporter genes in the viral strains is important for research purposes. EGFP allows easy monitoring of the viral particles in cell cultures by using a fluorescence microscope, whereas hLuc enables monitoring of the virus mutants *in vitro* as well as *in vivo* following their injection into animal models, using non-invasive bioluminescence imaging [53].

Oncolytic activity

The deletion of RR was aimed to increase the oncolytic activity of HSV-1 particularly on glioblastoma cells. Indeed, the viability of U87 cells infected with KOS-RR and Vhs-RR was very low compared to the effect of infection with KOS and Vhs-1 (Figure 5). The RR deletion mutant hrR3 also showed similar effects of cultured U87 cells, destroying all of them within 3 days after infection [42] and colon carcinoma cells [70].

Apoptosis

Regarding apoptosis, HSV-1 has a dual effect on infected cells. In some cases, apoptosis plays a role in the viral pathogenesis, like in the case of infection of the adrenal gland in mice with HSV-1 that resulted in apoptosis of the infected cells [5], or apoptosis of brain cells during encephalitis induced by infection with HSV-1 [8]. The induction of apoptosis by wild type HSV-1 occurs prior to six hours post-infection, and the novo viral protein synthesis is not required to induce the process [9]. On the other hand, cases were found when the wild type HSV-1 inhibited apoptosis of infected cells in order to facilitate its proliferation. Apoptosis of virus-infected cells occurs either as a direct response to viral infection or upon recognition of infection by the host immune response. Apoptosis reduces production of new virus from these cells, and therefore viruses have evolved inhibitory mechanisms to apoptosis in order to complete their

proliferation in the infected cells. This inhibitory effect on apoptosis upon infection with HSV-1 was observed in several cell types including human Hep-2 [9], Jurkat [29] and primary cultures of mouse cerebellar granule neurons (CGNs) [12]. It has been shown that accumulation of early (β) and leaky-late (γ 1) HSV-1 proteins correlates with the prevention of apoptosis in infected Hep-2 cells [10]. Several viral genes were found to be involved in the anti-apoptotic effect, like Us5, Us3 [30], ICP22, ICP27 [9], and the latency associated transcript (LAT) that was found to inhibit apoptosis probably by blocking caspase-3 and casapase-8 cleavage and activation [4, 15, 52]. Vhs and RR that were manipulated in the current study are also known as being involved in the inhibition of apoptosis. Vhs plays a major role in shutting-off protein synthesis in the infected cells, thus prevents them from starting apoptosis. Therefore, abolishment of the protein synthesis shut-off activity should allow induction of apoptosis by the infected cells. This has been shown with primary cultures of mouse CGNs. CGN cells infected with wild type HSV-1 were protected against apoptosis at least for a while, whereas infection with vhs-1 induced apoptotic cell death at earlier times [12]. On the other hand, the large subunit of HSV-1 RR was found to have a more direct effect on the apoptotic cascade by interfering at or upstream of caspase-8 activation [37]. Therefore, deletion of RR should result in apoptosis of the infected cells as has been shown with the RR deletion mutant hrR3 that increased apoptosis in infected PANC-1 cells upon infection [63]. In the current study we have shown that KOS, Vhs-1, KOS-RR and Vhs-RR caused apoptosis in infected lung cancer H1299 cell cultures, whereas U87 cells undergone apoptosis only after infection with Vhs-RR (Figures 6-9). Difference in the susceptibility of various cell lines to HSV-1-induced apoptosis were attributed to differences in the cellular apoptotic machineries [47, 48]. Our results indicate that the cellular machinery is not the only factor that determines the susceptibility of the infected cells to viral-induced apoptosis, but also the composition of viral proteins, based on the different apoptotic effects of Vhs-RR on U87 compared to the other viral strains. Nevertheless, the important phenomenon that was discovered in the current study is that only the double mutant Vhs-RR caused apoptosis in U87 GBM cells upon infection.

Viral growth

We have shown that the RR deletion mutants grows less effectively in vitro on Vero and U87 cells compared to their parental wild type strains. This was reflected in the viral yields on both cell lines (Figures 10 and 11; Table 2) and plaque size on Vero cells (Figure 12). Similar results were obtained with the RR deletion mutant ICP6 Δ , which also grew poorly compared to wild type HSV-1 on Vero cells as well as on murine and human cell cultures [14, 23].

When comparing the growth pattern of the four viral strains on Vero and U87 cells, it was found that the yields of KOS and Vhs-1 on U87 were lower than their yields on Vero cells, KOS-RR gave similar yields in both cell lines and Vhs-RR proliferated much better on U87 than on Vero. This behavior also makes Vhs-RR suitable for serving as an oncolytic vector for the treatment of GBM malignancies. In terms of safety we have shown that the wild type KOS strain grows well on non-dividing Goiter cells, whereas Vhs-1, KOS-RR and Vhs-RR barely grow on the non-dividing cells (Figures 13 and 14). These results emphasize the point that wild type HSV-1 cannot be used as an oncolytic vector for the treatment of cancer because its ability to proliferate in non-dividing cells, whereas the other three mutant strains lost

this ability, thus rendering them safer for the treatment of cancer especially in the CNS where the cancer cells are the only cell type that actively divide.

Summary

In the current study we have developed a double mutant of HSV-1 which is impaired in the virion host shutoff activity and deleted of RR. This mutant enables double function in cancer gene therapy: The ability to replicate only in actively dividing cancer cells while sparing normal non-dividing cells in one hand, and the ability to insert a foreign therapeutic gene. Currently we inserted two reporter genes instead of RR – EGFP and hLuc, which facilitate monitoring the viral vector in vitro and in vivo. These genes can be replaced with therapeutic genes.

Vhs-RR has an oncolytic effect on cultured U87 GBM cells, when one of its oncolytic mechanisms is induction of apoptosis in the infected cells. This double mutant was also found to grow less effectively on non-dividing cells than on actively dividing cells, rendering it safe for virotherapy in animal models and humans. This is the first combination of mutations in Vhs and RR of HSV-1, which has been shown to be a promising candidate for oncolytic treatment of brain tumor malignancies. In vivo experiments are underway aiming at evaluating the efficacy of Vhs-RR in xenografted human GBM tumors in animal models.

Acknowledgements

This manuscript is in loving memory of the late Prof. Niza Frenkel who passed away before her time.

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Figure 1: The RR-deletion plasmid – pNF-1282.

Figure 2: Deletion of UL39 and UL40 in the HSV-1 genome following homologous recombination in regions H1 and H2.

The positions of the primers used for PCR analysis are indicated.

Figure 3: PCR analysis of KOS, Vhs-1, KOS-RR and Vhs-RR for the detection of EGFP and hLuc genes.

Primers PS-18 and PS-20 were used to check the presence of EGFP and primers PS-21 and PS-19 for the presence of hLuc.

Figure 4: PCR analysis of KOS, Vhs-1, KOS-RR and Vhs-RR for the detection of UL39 and UL40 genes.

Primers PS-18 and PS-16 were used to check the presence of UL39 and primers PS-17 and PS-19 for the presence of UL40.

Figure 5: Effect of viral infection on cell viability of U87 glioblastoma cells, as determined by MTT assay. The absorbance ($A_{570}-A_{630}$) is proportional to the number of viable cells in the culture.

Figure 6: Apoptotic DNA degradation in H1299 and U87 cell cultures following infection with KOS, Vhs-1 and Vhs-RR, 1 day post-infection.

Figure 7: Apoptotic DNA degradation in H1299 and U87 cell cultures following infection with KOS, Vhs-1 and Vhs-RR, 2 days post-infection.

Figure 8: Apoptotic DNA degradation in HI299 and U87 cell cultures following infection with KOS, Vhs-1 and Vhs-RR, 3 days post-infection.

Figure 9: Apoptotic DNA degradation in H1299 and U87 cell cultures following infection with KOS-RR.

Figure 10: Infectious virus yield of wild type and mutant HSV-1 strains on Vero cells, at starting infection ratio of 5 PFU/cell.

Figure 11: Infectious virus yield of wild type and mutant HSV-1 strains on U87 cells, at starting infection ratio of 5 PFU/cell.

Figure 12: Plaque size of KOS, Vhs-1, KOS-RR and Vhs-RR on Vero cells.

A. Photographs of representative plaques; B. Average plaque areas.

Figure 13: Infectious virus yield of wild type mutant HSV-1 strains on actively growing Vero cells, at a starting infection ratio of 3 PFU/cell.

Figure 14: Infectious virus yield of wild type mutant HSV-1 strains on non-growing Goiter cells, at a starting infection ratio of 3 PFU/cell.