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REVIEW

Designing herpes viruses as oncolytics

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Oncolytic herpes simplex virus (oHSV) was one of the first genetically-engineered oncolytic viruses. Because HSV is a natural human pathogen that can cause serious disease, it is incumbent that it can be genetically-engineered or significantly attenuated for safety. Here, we present a detailed explanation of the functions of HSV-1 genes frequently mutated to endow oncolytic activity. These genes are nonessential for growth in tissue culture cells but are important for growth in postmitotic cells, interfering with intrinsic antiviral and innate immune responses or causing pathology, functions dispensable for replication in cancer cells. Understanding the function of these genes leads to informed creation of new oHSVs with better therapeutic efficacy. Virus infection and replication can also be directed to cancer cells through tumor-selective receptor binding and transcriptional- or post-transcriptional miRNA-targeting, respectively. In addition to the direct effects of oHSV on infected cancer cells and tumors, oHSV can be "armed" with transgenes that are: reporters, to track virus replication and spread; cytotoxic, to kill uninfected tumor cells; immune modulatory, to stimulate antitumor immunity; or tumor microenvironment altering, to enhance virus spread or to inhibit tumor growth. In addition to HSV-1, other alphaherpesviruses are also discussed for their oncolytic activity.

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Today there are many therapies to address the malady of cancer, although often with incomplete success. A confounding issue with conventional therapies is that they do not specifically target tumor cells, often destroying the normal untransformed cells of the patient in the process of treatment. Chemotherapeutics and radiation generally disrupt and kill any cell that is rapidly synthesizing DNA, tumor or not. Surgery removes tissues that appear to be tumors and often leaves behind malignant cells. These therapies lack the targeted and continual killing of tumor cells which oncolytic virus therapy offers. Oncolytic viruses (OV) selectively replicate in and kill tumor cells while sparing normal cells.[1] Oncolytic activity is endowed through multiple mechanisms: viruses with a natural selectivity for tumor cells (*i.e.*, myxoma and Newcastle disease viruses), vaccine vectors that have been genetically manipulated or attenuated (*i.e.*, measles and vaccinia viruses), and genetically-engineered viruses with mutations in pathogenic genes, genes required for replication in normal cells, and/or retargeted to tumor cell receptors (*i.e.*, adenovirus and herpes simplex virus (HSV)). Infecting a tumor with an OV leads to a positive feedback system, whereby an OV infects a tumor cell and produces more therapeutic virus to infect and kill more tumor cells, thereby spreading throughout the tumor while sparing normal tissues. OVs have advantages over chemotherapy, antibody, and radiation therapies because they are biological agents capable of renewing themselves whilst spreading throughout the tumor.

Most viruses are very small 10–200 nm particles and all reproduce their genetic material during infection of a host cell. A virus is unable to replicate by itself and thus hijacks the host's transcriptional and translational machinery in order to create more virions before exiting and/or killing the host cell. Tumors are made up of dividing cells with diminished control of normal cell cycle/cell death and biosynthesis regulation, allowing them to endlessly proliferate. The increased levels of DNA and protein synthesis required for dividing tumor cells, compared to normal nondividing cells, makes them more permissive for productive viral replication.

The HSV-1 virion is composed of an envelope, a tegument, and a viral capsid core. The envelope is studded with viral glycoproteins responsible for grasping onto host attachment factors heparin sulfate, nectin-1, PILRα, and herpesvirus entry mediator (HVEM), and inducing membrane fusion.[2] The tegument is made up of multiple HSV-1 proteins, including ICP0, ICP4, and Us11, which are freed into the cytosol to prepare the cell for virus replication.[2] The capsid is transported to the cell's nucleus where viral replication will begin. HSV-1's lytic lifecycle is composed of three sequential steps. First, α or immediate early (IE) genes are transcribed into mRNAs and translated. The five IE proteins (ICP0, ICP4, ICP22, ICP27, and ICP47) are responsible for gene regulation, disabling certain innate and adaptive immune functions, as well as enabling the virus to move to the next step in its lifecycle.[2] Once the IE ICP4 protein is recruited to the virus genome, β or Early genes (E) are transcribed. Many of the E genes are involved in the replication of the virus's DNA genome. Discrete compartments form in the nucleus of infected cells where viral DNA replication occurs. The final step in the virus lifecycle is the Late (L) step, whereby the L or γ genes are transcribed and translated after viral DNA synthesis. The new viral genomes are packaged

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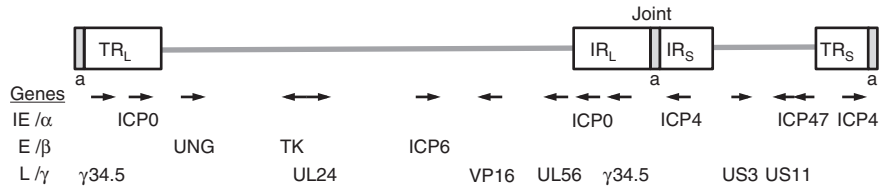

Figure 1 Schematic map of herpes simplex virus (HSV) genome illustrating the position of genes (IE, E, L) deleted or mutated in oHSVs. The genome consists of unique long (L) and short (S) sequences bracketed by terminal (TR) and internal (IR) inverted repeat sequences and separated by the joint region. The number of *a* sequence repeats is variable. Arrows indicate direction of transcription.

in virions that are exported from the nucleus to the cell membrane in order to spread to new cells. The entire lytic cycle takes between 8 and 24 hours from infection to exit. HSV-1 is also capable of establishing a lifelong latent (nonlytic) infection in peripheral neurons. Latency is defined by the absence of viral protein production, until a reactivation event occurs causing the virus to rouse itself from dormancy and spread to other cells.[2]

The HSV-1 genome is around 150 kilobases in length and encodes about 84 genes.[2] Unlike smaller viruses, HSV-1 encodes many genes that are not essential for its productive growth in cultured cells. This allows researchers to manipulate the HSV-1 genome to enhance oncolytic activity without critically damaging the ability of the virus to replicate. In order to create oncolytic HSVs (oHSVs), it is important to understand the functions of the gene(s) to be altered; their contributions toward cell specificity, replication efficiency and pathology, and whether or not removing them will fundamentally alter the virus's ability to replicate. In this review we discuss the function of viral genes that have been altered to create oHSVs, and additional strategies to target tumor cells and enhance oncolytic activity.

GENE MUTATIONS IN HSV-1 ENDOWING ONCOLYTIC ACTIVITY

The HSV-1 genome has many genes that can be deleted or mutated to confer safety and/or tumor targeting specificity (Figure 1). In this section, we will discuss the function of genes mutated in oHSV, what roles they play in the replication cycle of the virus, and how mutations in these genes confer oncolytic activity. The genetic alterations of oHSVs are described in Table 1. The parenthesized letters (IE, E, L) indicate when the gene is expressed during the HSV-1 lifecycle. The key distinction to be made when designing an oHSV is: what is the difference between the tumor cells to be destroyed and the normal cells to be spared?

NUCLEOTIDE METABOLISM

One general feature of cancer cells that distinguishes them from the majority of normal cells, which are postmitotic, is that they proliferate, and thus maintain sufficient nucleotide pools for DNA replication. HSV-1 harbors several genes involved in nucleotide metabolism (thymidine kinase, ribonucleotide reductase, uracil DNA glycosylase) that permit virus replication in nondividing cells lacking sufficient nucleotide pools. Mutations in these genes confer specificity for dividing cells and often attenuate viral pathogenicity as well.

Thymidine kinase/UL23 (E)

HSV-1 thymidine kinase (TK) is a multifunctional enzyme that catalyzes the creation of deoxythymidine 5′-phosphate (dTMP) from deoxythymidine, as well as phosphorylating deoxycytidine and nucleoside analogs, creating precursors for viral DNA synthesis.[2] Loss of TK activity halts HSV-1 replication in nondividing cells, making TK mutants safer than wild type HSV-1.[3] However, the antiherpetic nucleoside analog drugs, *i.e.*, acyclovir and ganciclovir, depend upon TK for inhibition of DNA replication. Therefore, oHSVs without TK are more difficult to treat should a dangerous expansion of the virus occur in a patient undergoing oHSV therapy. The availability of antivirals that do not depend upon TK, but directly target HSV DNA polymerase, like foscarnet and cidofovir, even with their intravenous administration provides a basis for reconsidering TK$^-$ oHSV.[4] Conversely, TK has been used as a "suicide gene" in oHSV by adding ganciclovir during viral infection (see cytotoxic transgenes).[5]

*Dl*sptk, a HSV-1 lacking TK, was the first genetically-engineered oncolytic virus devised to kill tumor cells while sparing normal cells.[6] Originally created to study TK function in HSV-1, *dl*sptk selectively killed U87 glioblastoma cells and increased the survival of nude mice harboring U87 orthotopic gliomas, validating the hypothesis that mutations in viral nucleotide metabolism could endow HSV-1 with tumor selectivity.[6] Other TK-deleted oHSVs (*Dl*8.36tk, KOS-SB) were found to be efficacious in immunocompetent rat tumor models.[7,8] One drawback of *dl*sptk, was that it was insufficiently attenuated in the brain.[6]

ICP6/UL39 (E)

ICP6 is the large subunit of the viral ribonucleotide reductase, which transforms ribonucleotides into deoxyribonucleotides (dNMP) by reducing the 2′-COH group.[2] HSV-1 requires an abundant supply of dNMPs to synthesize new viral genomes. ICP6 is essential for HSV replication in noncycling cells, making ICP6-null oHSVs selective for dividing cells.[9] Quiescent cancer cells that are mutated for p16^{INK4a} are also permissive for ICP6$^-$ HSV-1, suggesting ICP6$^-$ oHSVs could target quiescent tumor stem cell populations with mutations in their cell cycle control.[10] In relation to safety, ICP6 deleted HSV-1 is attenuated in mice compared to wild type HSV-1.[11,12] This is attributed to the inability of the virus to replicate in and kill mature neurons and other postmitotic cells in the brain, which lack ribonucleotide reductase expression and sufficient pools of dNTPs for virus replication.[13] However, it has not been reported how large a dNTP pool is needed to support HSV-1 replication. Interestingly, even in cancer cells, increasing cellular ribonucleotide reductase activity, through the use of ENT1 inhibitors, or 5FU or FUdR, can increase the replication of ICP6$^-$ oHSV.[14–16] For example, an approximate 30% increase in ribonucleotide reductase activity after ENT1 inhibition with dilazep resulted in a greater than 10-fold increase in virus yield.[14] Mutating ICP6 in oHSV restricts the virus to cycling cells, thus giving the virus selectivity for tumor cells. Likewise, mutations within the ICP6 gene also prevent oHSV from harming normal tissue.

ICP6, through its N-terminus directly binds eIF4G, expediting the formation of the eIF4 protein translation complex via eIF4E phosphorylation, and stabilizes interactions between eIF4G and eIF4E.[17] However, the N-terminal domain alone, as in hrR3, does not promote complex formation.[17] EIF4 complex formation is often

Table 1 Oncolytic HSV constructs

Oncolytic virus	*HSV gene mutations (deletion: Δ; inactivating mutation: -)*	*Transgenes*	*Reference*
1716*	γ34.5Δ		182
1716-6	γ34.5Δ, ICP6-	ICP6-LacZ fusion in ICP6	23
3616UB	UNG (UL2)-, γ34.5Δ	UNG-LacZ fusion in UNG	27
C134	γ34.5Δ	CMVpro-CMV IRS1	52
Δ68H-6	γ34.5 BBDΔ, ICP6-	ICP6-LacZ fusion in ICP6	23
ΔPK	HSV-2; ICP10-PKΔ		96
*Dl*8.36tk	TK-	LacZ	7
*Dl*sptk	TKΔ		6
FusOn-H2	HSV-2: ICP10-PKΔ	CMVpro-eGFP-ICP10 RR fusion	97
G207*	γ34.5Δ, ICP6-	ICP6-LacZ fusion in ICP6	22
G47Δ*	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	ICP6-LacZ fusion in ICP6	55
G47ΔUs11fluc	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	Us11pro-fluc, ICP6pro-LacZ in ICP6	183
HF10*	IRLΔ, UL56-LATΔ, gB^{syn}, UL53-55 duplicated		85
hrR3	ICP6-	ICP6-LacZ fusion in ICP6	25
JD0G	ICP0Δ, Joint deletion (1 copy of ICP4, γ34.5)	CMVpro-eGFP in ICP0	83
KM100	$ICP0^{n212}$, $VP16^{in1814}$		88
KM110	$ICP0^{n212}$, $VP16^{V422}$		88
L1BR1	HSV-2; Us3Δ	ICP8pro-LacZ in Us3	77
MG18L	Us3Δ, ICP6-	ICP6-LacZ fusion in ICP6	24
NV1020* (R7020)	UL24Δ, UL5, 6 (duplicated), TKΔ, UL56Δ, Joint deletion (1 copy of ICP0, ICP4, γ34.5)	ICP4pro-TK, HSV-2: gG, PK, in joint	87
NV1023	UL24+, UL5, 6 (duplicated), TK+, UL56Δ, Joint deletion (1 copy of ICP0, ICP4, γ34.5), ICP47Δ, Us11Δ, Us10Δ	ICP47pro-LacZ	87
NV1066	Joint deletion (1 copy of ICP0, ICP4, γ34.5) copy), TKΔ	CMVpro-GFP in Joint, BAC in TK	184
R3616	γ34.5Δ		42
R7041	Us3Δ		69
SUP1	γ34.5Δ, ICP47Δ, ICP47pro-Us11	γ34.5pro-Gluc	57
Targeted vectors			
bM24-TE	ICP4Δ, US3-, ICP6-	4F2 enh-TRE-CMVminpro-ICP4, ICP6-LacZ fusion in ICP6	107
CMV-ICP4-143T /-145T (amplicon plasmid)	CgalΔ3 (ICP4Δ) helper HSV	miR 143 or 145 targeting sequence in ICP4	106
D12.CALP	ICP4Δ, TK-, UL24Δ, US3-	TKpro-LacZ, 4F2 enh-Calponin pro-ICP4 in TK	108
G92A	ICP4Δ, TK-, UL24Δ, US3-	TKpro-LacZ, Albumin enh-pro-ICP4 in TK	90
KeM34.5	γ34.5Δ, ICP6-	Msi pro-γ34.5 in ICP6	118
KGE4:T124	Deletion of residues 2-24 and amino acid substitution, Y38C, in gD. gB:NT	αhuEGFR scFv in gD2-24Δ, gC-GFP fusion, miR-124 target in ICP4 3'UTR	120
KNC	Deletion of residues 2-24 and amino acid substitution, Y38C, in gD. gB:NT	αhuCEA scFv inserted in gD2-24Δ	127
KNE	Deletion of residues 2-24 and amino acid substitution, Y38C, in gD. gB:NT	αEGFRscFv inserted in gD2-24Δ	127
LCSOV	gHΔ	ApoE-AATpro-gH-miR112a, miR124a, miRlet7 targets in gH	121
Myb34.5	ICP6Δ, γ34.5Δ	Myb pro-γ34.5 in ICP6	113
oHSV-MDK-34.5	γ34.5Δ, ICP6Δ	Mdk pro-γ34.5 and ICP6-GFP fusion in ICP6	119

Table 1 Continued on next page

Table 1 Continued

Oncolytic virus	*HSV gene mutations (deletion: Δ; inactivating mutation: -)*	*Transgenes*	*Reference*
R5141	Deletion of residues 68-78 in gB, 1-140 in gC, and 1-33 in gD. gC V34S substitution	IL13 fused to gD and gC	125
R-LM113	Deletion of residues 6-38 in gD	scHER2 fused to gD, ICP27pro-EGFP between UL3 and UL4	130
R-LM249	Deletion of residues 61-218 in gD	scHER2 fused to gD, ICP27pro-EGFP between UL3 and UL4	126
rQnestin34.5	γ34.5Δ, ICP6Δ	Nestin enh-Hsp68pro-γ34.5 and ICP6-GFP fusion in ICP6	48
Armed vectors			
bPΔ6-hPAP	ICP6Δ	CMVpro-hPAP, ICP6-LacZ in ICP6	136
34.5ENVE	γ34.5Δ, ICP6Δ	ICP6-GFP fusion, IE4/5pro-Vstat120, Nestin enh-Hsp68pro-γ34.5 in ICP6	117
G47Δ-IL18/B7	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	CMVpro-mIL-18-IRES-B7-1-Ig, ICP6-LacZ fusion in ICP6	133
G47Δ-mIL12	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	CMVpro-mIL-12, ICP6-LacZ fusion in ICP6	140
G47Δ-mAngio	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	CMVpro-smAngio, ICP6-LacZ fusion in ICP6	173
G47Δ-PF4	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	CMVpro-PF4, ICP6-LacZ fusion in ICP6	175
HSV1790	γ34.5Δ	CMVpro-NTR-IRES-eGFP	151
HSV-1γCD	ICP6-	CMVpro-yeast CD, CMVpro-AFP in ICP6	147
HSV-PNP (M016)	γ34.5Δ	Egr1pro-PNP in γ34.5	152
M002	γ34.5Δ	Egr1pro-mIL-12 in γ34.5	166
M012	γ34.5Δ	Egr1pro-bacterial CD in γ34.5	148
M032*	γ34.5Δ	Egr1pro-hIL-12 in γ34.5	169
MGH2	γ34.5Δ, ICP6-	HSVIE4/5pro-CYP2B1, CMVpro-shiCE, ICP6-GFP fusion in ICP6	150
NV1034	UL5, 6 (duplicated), UL56Δ, Joint deletion (1 copy of ICP0, ICP4, γ34.5), ICP47Δ, Us11Δ, Us10Δ	ICP47pro-LacZ in ICP47, ICP4 enh-TKpro-GM-CSF, HSV-2 gG, PK in joint	87
NV1042	UL5, 6 (duplicated), UL56Δ, Joint deletion (1 copy of ICP0, ICP4, γ34.5), ICP47Δ, Us11Δ, Us10Δ	ICP47pro-LacZ in ICP47, ICP4 enh-TKpro-mIL-12, HSV-2 gG, PK in joint	87
oHSV-NIS	γ34.5Δ, ICP6-	CMVpro-NIS in ICP6, ICP6-GFP fusion	145
oHSV-TRAIL	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	IE4/5pro-sTRAIL in ICP6, ICP6-LacZ fusion	155
OncoVex^{GMCSF}* (Talimogene laherparepvec)	γ34.5Δ, ICP47Δ	CMVpro-GM-CSF in γ34.5	56
OV-Chase	γ34.5Δ, ICP6-	IE4/5pro-Chase, ICP6-GFP fusion in ICP6	179
R8308	γ34.5Δ	Egr1pro-mIL-10 in γ34.5	171
RAMBO	γ34.5Δ, ICP6-	ICP6-GFP fusion, IE4/5pro-Vstat120 in ICP6	176
rQT3	γ34.5Δ, ICP6-	IE4/5pro-TIMP3, ICP6-GFP fusion in ICP6	180
rRp450*	ICP6Δ	ICP6pro-CYP2B1 in ICP6	149
Synco-B18R	γ34.5Δ, syn-,	UL38pro-B18R, CMVpro-eGFP in joint	53
T-TSP-1	γ34.5Δ, ICP6-, ICP47Δ, ICP47pro-Us11	CMVpro-TSP1, ICP6-LacZ fusion in ICP6	177
VAE	γ34.5Δ, ICP6-	ICP6pro-endo-angio fusion in ICP6	174

AFP, aequorea fluorescent protein; BAC, bacterial artificial chromosome; BBD, beclin binding domain; CEA, carcinoembryonic antigen; CD, cytosine deaminase; Chase, chondroitinase ABC; CYP2B1, cytochrome P450; eGFP, enhanced green fluorescent protein; endo-angio, endostatin-angiostatin; enh, enhancer; fluc, firefly luciferase; gD, HSV glycoprotein D; Gluc, β-glucuronidase; h, human; IE4/5, immediate-early gene 4/5; IL, interleukin; LacZ, β-galactosidase; m, mouse; minpro, minimal promoter; Msi, musashi1; NIS, sodium/iodide symporter; NTR, E. coli nitroreductase; PNP, E. coli purine nucleoside phosphorylase; PK, protein kinase; pro, promoter; RR, ribonucleotide reductase; TIMP3, tissue inhibitor of metalloproteinases 3; TK, thymidine kinase; shiCE, secreted human intestinal carboxylesterase; smAngio, secreted mouse angiostatin; sTRAIL, secreted TNF-related apoptosis-inducing ligand; TRE, Tcf response element; TSP1, thrombospondin-1; Vstat120, vasculostatin.
oHSVs in clinical trial are marked with *.

reduced in growth-arrested cells so capped mRNAs, including HSV transcripts, are poorly translated. Thus, tumor cells lacking negative regulation of their initiation complexes will be susceptible to ICP6 mutant oHSV, while normal cells will shut down viral protein production. In addition, the ICP6 N-terminal RHIM domain binds and activates RIP3, triggering necrosis.[18] ICP6 also plays a role in blocking TNFα- and FasL-induced apoptosis, likely through interactions with caspase 8 and RIP1.[19,20] This antiapoptotic effect was not seen with hrR3, suggesting the inhibitory domain may be in the C-terminus.[20] These actions confer further specificity to tumor cells, which tend to be resistant to apoptosis. As additional safety features, ICP6 mutants are somewhat temperature-sensitive and hypersensitive to antiviral nucleoside analogs and DNA replication inhibitors.[9,21]

Aside from the colon crypts, epidermal and bone marrow progenitors, and activated splenic immune cells, most normal tissues in humans are not actively replicating. Removing ICP6 limits the ability of the HSV-1 to replicate in postmitotic cells and directs the virus toward dividing tumor cell populations. For these reasons, deletion/inactivation of ICP6 creates a safer oHSV while targeting actively growing tumors. ICP6-deleted rRp450 is currently in a clinical trial for liver tumors (see http://clinicaltrials.gov/show/NCT01071941). ICP6 mutations have been combined with other single mutations in order to enhance safety and tumor specificity; G207 from R3616,[22] 1716-6 from 1716,[23] MG18L from R7041,[24] and Δ68H-6 from Δ68H[23] (Table 1). The C-terminus of ICP6 is responsible for its ribonucleotide reductase activity, which is why many oHSVs have inserted a LacZ fusion within this region of the gene to inactivate it.[22–25] The N-terminus of ICP6 is responsible for expediting the eIF4G interaction with eIF4E and binding RIP3, and is present in several oHSVs. Going forward, it will be important to ascertain which functions of ICP6 are related to oncolytic activity.

UL2/UNG (E)

UL2, uracil DNA glycosylase, is another HSV-1 gene involved in nucleotide metabolism, which when removed attenuates neurovirulence, neuroinvasiveness, and reactivation.[26] Uracil-DNA glycosylases prevent mutagenesis by excising uracil from DNA molecules and signaling the base excision repair pathway. OHSV 3616UB lacks γ34.5 and UL2, and is hypersensitive to ganciclovir.[27] It demonstrated oncolytic efficacy in six brain tumor cell lines as well as in xenografts, and could not replicate in mature neurons *in vitro* or *in vivo*.[27]

PATHOGENICITY AND ANTIVIRAL SUPPRESSION

Mammalian organisms have evolved multiple mechanisms for detecting and disabling intracellular pathogens. In order for HSV-1 to replicate its genome and create the next generation of virus, it must cloak itself or actively disrupt host responses to foreign pathogens. Many of the HSV-1 genes which inhibit the host cell's ability to initiate apoptosis or hamper viral replication are nonessential for viral replication in cancer cells, and therefore provide mutation targets for creating oHSV.

γ34.5/RL1 (L)

γ34.5, one of the most frequently mutated genes for creating oHSVs, has many functions. HSV-1 is diploid for the γ34.5 gene, and mature virions contain the protein in their tegument in order to deliver it upon entry.[2] γ34.5 promotes the rapid dephosphorylation of the translational initiation factor eIF2α by activating protein phosphatase 1 (PP1α).[28] When eIF2α is phosphorylated, eIF2β is unable to exchange GDP for GTP and methionine-tRNA is not loaded into the ribosome, preventing translation from initiating.[29] Dephosphorylation of eIF2α prevents host translational shutoff induced by virus infection, thereby allowing the continued production of viral proteins.[28] EIF2α is phosphorylated by several kinases (PKR, PERK, GCN2, HRI) activated by environmental stresses or upon detection of foreign pathogen associated molecular patterns (PAMPs) such as viral DNA and double-stranded RNA, and dephosphorylated by PP1α bound to GADD34.[29] The C-terminus of γ34.5 is homologous to GADD34 and can replace GADD34 for PP1α activation.[30] Thus, treatment with chemotherapeutics that induce GADD34 expression can enhance γ34.5-deficient oHSV replication and cytotoxicity.[31–33] Tumor cells often lack the meticulous control of translation or antiviral/stress responses observed in nontransformed cells, allowing oHSV lacking γ34.5 to replicate. Many tumors also overexpress eIF2α, coinciding with the down regulation of the eIF2 kinases, effectively disabling the tumor's ability to halt viral protein translation, even in the absence of γ34.5.[29]

In addition to disrupting the translational shutoff pathway, γ34.5 interrupts autophagic vesicle maturation,[34] blocks the tank binding kinase (TBK-1)-mediated interferon-stimulated gene (ISG) signaling pathway,[35] interacts with proliferating cell nuclear antigen (PCNA),[36] and relocates p32 (HABP1/gC1qR) for nuclear egress.[37] Autophagic vesicles, or autophagosomes, are created by a collection of autophagy factors and kinases supported by the mitochondrial protein Beclin-1 (BCN1; yeast homologue Atg6).[38] BCN1 binds to PI3 kinase class III (PI3KcIII) and other ATG co-factors, to convert LC3-I into LC3-II and recruit ATG components into forming autophagic vesicles.[38] γ34.5 disrupts autophagosome formation by binding to BCN1 via its BCN1 binding domain (BBD; amino acids 68-87), preventing BCN1 binding to ATG/PI3KcIII cofactors.[39] Autophagy has both positive and negative impacts on tumorigenesis and maintenance,[38] but γ34.5 BBDΔ oHSV (Δ68H-6) replicates in and kills glioma cells similarly to γ34.5+ oHSV.[23] Deletion of the γ34.5 BBD somewhat attenuated neuropathogenicity.[39,40] Therefore, ICP6 inactivation was combined with BBD deletion in Δ68H-6 to eliminate pathogenicity.[23] TBK-1 normally functions as a messenger protein to activate IRF3, which then activates interferon α/β and ISGs. γ34.5 binds TBK-1, preventing it from activating the IRF3 complex.[35] Thus, HSV lacking γ34.5 are highly susceptible to interferon (IFN) inhibition. The function of γ34.5 binding to PCNA and its shuttling between the nucleus and cytoplasm is unknown.[36,41] P32 relocalizes to the nuclear envelope upon binding γ34.5 to facilitate nuclear egress, and knockdown of p32 decreased the replication of the virus.[37]

The γ34.5 gene is the major determinant of HSV neuropathogenicity, although the exact mechanism(s) by which γ34.5 contributes to herpes simplex encephalitis is unclear.[42] MHC class II cell surface complexes are increased after infection of glioma cells with γ34.5-deleted R3616, as compared to wild-type or mock infection.[43] γ34.5-deleted HSV-1 regains neurovirulence in IFNα/βR- or PKR-deficient mice.[44] Thus, many oHSVs being clinically evaluated have both copies of γ34.5-deleted: G207, G47Δ, 1716, OncoVEX^{GMCSF}, and M032 (Table 1). Unfortunately, γ34.5-deleted oHSVs (1716, G207, R3616), but not G47Δ or Δ68H-6, replicate poorly, if at all, in glioblastoma stem cells.[23,45] oHSVs containing only 1 copy of γ34.5 have been constructed (*i.e.*, NV1020), but the contribution of the γ34.5 deletion is unclear because of other mutations present in these oHSVs. To improve safety and reduce the chance of second-site suppressors, deletion of γ34.5 was combined with ICP6 inactivation to create G207, the first oHSV to enter clinical trial in the US.[22,46,47] Because deleting γ34.5 attenuates virus replication

even in cancer cells, a strategy to confine γ34.5 expression to cancer cells was developed. OHSV rQnestin34.5, with the endogenous γ34.5 genes deleted, has a γ34.5 gene cassette controlled by a nestin promoter so it is only expressed in cancer cells expressing nestin[48] (see Transcriptional Targeting). An unbiased strategy has been to select more effective oHSV by passaging virus in tumors or tumor cells. The ICP47 deletion (see below) was isolated by passaging γ34.5-deficient HSV-1 in non-permissive SK-N-SH tumor cells.[49] This second-site suppressor of γ34.5 was due to immediate-early expression of Us11 because of deletion of its late promoter.[50] A similar strategy was followed by passaging M002 in D54MG glioma cells in culture or glioma xengrafts. The *in vivo* passaged virus was more efficacious in both immune-deficient D54MG and syngeneic Neuro2a brain tumor models, while the *in vitro* passaged virus had increased neurovirulence.[51] However, no analysis was performed to identify the genetic alterations.

An alternate strategy to complement the loss of γ34.5 is to express genes from other viruses that evade PKR-mediated protein shutoff or IFN-mediated antiviral responses. CMV IRS1, which prevents PKR activation, has been inserted into γ34.5Δ R3616 to generate C134.[52] C134 infection of glioma cells, compared to its γ34.5Δ parent, overcame the block in protein synthesis, replicated better in tumors, and was more efficacious in extending survival of glioma-bearing mice, but maintained safety after intracranial injection.[52] Vaccinia virus B18R, a secreted IFNα/β decoy receptor, is expressed in Synco-B18R under control of the Late UL38 promoter, so it is only expressed in tumor cells after oHSV replication.[53] Synco-B18R was able to replicate in tumor cell lines poorly permissive to oHSV and was somewhat more efficacious *in vivo*, but was safe after intravenous administration.[53]

ICP47/Us12 (IE) + Us11 (L→IE)

ICP47 blocks the TAP protein channel preventing peptide loading onto MHC I protein and subsequent MHC I expression.[2] This shrouds virus-infected cells from HSV-1-specific $CD8^+$ T-cells, but can trigger an NK cell response due to the lack of MHC 1 on the infected cell's surface.[54] The TAP channel blocking activity of ICP47 is species-specific, making studies of ICP47 activity in mice not representative of the protein's activity in humans. ICP47 is deleted in G47Δ and several other oHSVs ($OncoVEX^{GMCSF}$, SUP-1 (Table 1)) to enhance immune responses against virus-infected tumor cells and antitumor immunity.[55–57] Deletion of the ICP47 ORF and Us11 promoter places Us11 under control of the ICP47 IE promoter. Us11 is a true L gene that binds dsRNA-dependent eIF2α kinase (PKR), precluding it from binding dsRNA and phosphorylating eIF2α.[58,59] IE expression of Us11 complements the deletion of γ34.5 in nonpermissive cells by keeping eIF2α dephosphorylated and protein translation unhindered. G47Δ and SUP-1 replicate in cells nonpermissive to γ34.5-deleted HSV and better in permissive cells, yet retain a lack of pathogenicity.[55,60]

RIGI/MDA5 are sensors that detect RNA in the cytoplasm and activate IRF3, leading to induction of the ISG pathway. Us11 directly binds RIGI and MDA5 preventing activation of IRF3 and production of IFNβ.[61] This interaction is RNA independent and mediated by the C-terminus of Us11, which is also where the PKR and dsRNA binding pockets of the protein are located.[61,62] Us11 excels at deactivating RNA-mediated immune responses, so that expression of Us11 rescues Sindbis virus replication, which is very sensitive to the RNA sensor activated ISGs.[61] Us11 also directly binds ISG 2′5′ oligoadenylate synthase (2′5′OAS) to prevent it from recognizing dsRNA and subsequently activating RNaseL.[63] Interruption of 2′5′OAS signaling allows the virus to express its mRNAs without being shut off by host defenses. Us11 also prevents cell death from staurosporine, associates with nucleolin within the nucleus, interacts with nuclear transport machinery during viral replication, and moderately inhibits autophagy.[62,64,65] How or whether these other functions of Us11 enhance the replication of γ34.5-deficient oHSV is unclear, however, IE expression of Us11 rescues the growth of γ34.5-deficient virus in nonpermissive tumor cells, while still being safe in mice.[55,60]

Us3 (L)

Us3 is a serine-threonine protein kinase with substrate specificity overlapping PKA and Akt,[66,67] affecting a multitude of antiviral host responses. One of the earliest functions identified was prevention of apoptosis in infected cells, possibly by phosphorylating PKA substrates, blocking proapoptotic BAD protein activation, and/or eliciting a prosurvival signaling pathway.[68] Defects in apoptotic pathways are common in tumor cells, while inhibition of apoptosis is important for virus replication in normal cells. Thus, Us3-deleted R7041 induced much greater apoptosis in normal HUVEC than its wild-type revertant, while inducing similar low levels in cancer cells.[69] Us3 phosphorylates a large number of viral and cellular proteins, affecting many pathways in infected cells: dUTPase (UL50) to increase enzymatic activity[70]; gB to enhance virus replication *in vivo*[71]; p65 to inhibit NF-κB activity and innate immune responses[72]; IRF3 to inhibit IFNβ expression[73]; IFNγRα to abrogate expression of IFNγ-dependent genes[74]; and TSC2 to activate mTORC1 and stimulate translation.[67] It also indirectly downregulates cell surface expression of MHC 1, increasing HSV-specific CD8+ T cells in mice.[75] These activities promote Us3-deleted oHSVs tumor specificity due to deficiencies in innate immune responses, as well as contribute to reducing neuroinvasiveness, neuropathogenicity, and peripheral pathogenicity of Us3-deleted oHSV.[76]

Us3-deleted HSV-1 (R7041) and HSV-2 (L1BR1) were shown to have oncolytic activity, with greatly reduced replication compared to wild-type HSV in normal cells *in vitro*, and elevated replication in tumors *in vivo* compared to normal tissue.[69,77] Chemotherapy increased apoptosis in L1BR1, but not wild-type, infected cancer cells.[77] R7041 was safe after intravenous injection in immune-deficient mice and intraperitoneal injection in immune-competent mice.[69] However, it was insufficiently safe in the brain, in contrast to γ34.5Δ virus, so MG18L containing an additional inactivating LacZ insertion in ICP6 was constructed.[24] MG18L induces increased levels of apoptosis in glioblastoma stem cells (GSCs) *in vitro* and *in vivo*.[24] Because Akt activation is elevated after infection with Us3-deleted HSV, the combination with PI3K/Akt inhibitors was evaluated and found to synergize in cancer cell lines and GSCs, through increased apoptosis.[24,69]

ICP0 (IE)

ICP0 encodes a RING finger ubiquitin E3 ligase that targets cellular proteins.[78] ICP0 targets PML or ND10 bodies and other host factors designed to silence HSV-1 genomes and induce IFN signaling for ubiquitination and degradation.[78] Thus, ICP0-null HSV-1 is extremely sensitive to IFN and PML-mediated disruption of the viral lifecycle.[78] In multiple tumors, PML is downregulated, making them permissive for ICP0-null oHSV.[79] ICP0 is also involved in 4E-BP1 degradation and promoting eIF4E phosphorylation, facilitating eIF4F complex formation in quiescent cells, important for HSV replication.[80]

oHSV KM100 has mutations which disable the ICP0 gene, as well as VP16.[81] It is able to grow in IFN-deficient breast cancer cell lines and demonstrated therapeutic efficacy using the

immunocompetent mouse FVB breast cancer model.[81] ICP0 truncation mutant HSV-1 (n212) replicated better in mouse cancer cells *in vitro* than KM100, while the efficacy of the two was the same in an immunocompetent mouse tumor model.[82] Mice receiving KM100 that survived the initial tumor were protected from tumor rechallenge, demonstrating that the virus elicits a memory immune response to the tumor.[81,82] Another ICP0-null oHSV, JD0G replicates in osteosarcoma U2OS and glioblastoma U251 cells.[83] The ability of ICP0 mutants to replicate in a variety of human and mouse tumor cell lines suggests that transformed cells complement ICP0 defects, while untransformed cells remain nonpermissive, making ICP0 a good target for designing oHSVs with specificity for tumor cells.

UL56 (L)

UL56 is another gene associated with pathogenicity and neuroinvasiveness, as removing it attenuates the virus.[84] oHSV HF10 has a number of deletions and insertions in the genome, resulting in the lack of expression of UL43, UL49.5, UL55, and UL56.[85] HF10 has demonstrated efficacy in a variety of preclinical models and entered early phase clinical trials for breast and head and neck cancer.[86] NV1020, which was initially developed as a vaccine (R7020), and NV1023 are also deleted for UL56, along with ICP47 and a single copy of ICP0, ICP4, and γ34.5.[87] Whether UL56 contributes to tumor selectivity is unknown, but it is a good candidate for mutation when creating a safer oHSV.

OTHER HSV-1 FUNCTIONS

The proteome of HSV-1 is rather well defined due to extensive study; however, the functions of many proteins still remain unknown. Because of the large genome size and number of nonessential viral genes, the opportunities for identifying additional genes endowing oncolytic activity remains. Some mutations have been incorporated into oHSV, without their contribution being defined. The following are other mutated genes present in oHSVs, whose roles are not well defined in relationship to oncolytic activity.

VP16/UL48 (L)

VP16 is delivered during entry of HSV-1 via the tegument. It plays a role in aiding the transcription of IE genes during initial infection and during reactivation from latency.[2] In KM100, VP16 is mutated along with ICP0.[88] Interestingly, combining a VP16 mutant lacking the C-terminus with ICP0-null (KM110), produces a less cytopathic, more debilitated virus than with VP16 mutation in1814 (in KM100), which removes the trans-activating activity of VP16.[88] This difference was also exhibited in cancer cell lines.[81]

UL24 (L)

The UL24 gene overlaps TK, so some TK deletions also contain UL24 mutations (*i.e.*, RH105),[89] although not *dl*sptk, and G92A.[90] UL24 causes the dispersal of nucleolin throughout the nucleus.[91] UL24 single mutants are defective in establishing latency and reactivation, replicating in the eye, and causing ocular disease.[92] UL24 mutants can also have a syn phenotype, whereby infected cells form syncytia.[2,92] NV1020 contains a UL24 deletion as a result of removing TK, as well as deletion of UL56.[87] NV1020 is efficacious in a variety of colon, gastric, and prostate cancer models and safe in patients, although the role of the UL24 deletion in its oncolytic activity is unknown.[93]

OTHER ONCOLYTIC HERPESVIRUSES

HSV-2

HSV-2, another human alphaherpesvirus primarily associated with genital disease, has a genome that is very similar to HSV-1, both in genomic organization and protein coding sequences.[2] In a comparison between ICP0Δ HSV-1 and HSV-2, HSV-2 dICP0 was more cytopathic in mouse breast cancer cells than HSV-1 dICP0, but had much lower virus burst sizes.[94] *In vivo*, in an immunocompetent mouse breast tumor model, only HSV-1 dICP0 significantly extended survival.[94]

The HSV-2 UL39 gene encodes ICP10, a homolog of HSV-1 ICP6 that contains a unique N-terminal domain proposed to encode a serine/threonine protein kinase (PK).[95] Thus, in addition to ribonucleotide reductase activity, ICP10 is a constitutively active growth factor receptor that directly interacts with RAS-GAP to activate RAS-GTP and stimulate the RAF/MEK/ERK signaling pathway, which prevents apoptosis in infected cells.[95] HSV-2 ICP10 PK deletions have been tested for oncolytic efficacy due to the inability of HSV-2 to replicate in noncycling cells without ICP10/PK, and the presence of constitutive RAS signaling and defective apoptosis in many tumors. FusOn-H2 and DeltaPK (HSV2ΔPK) are oHSV2 which have deletions of the PK domain of ICP10.[96,97] Both do not replicate in noncycling normal cells, induce apoptosis, and engender stalwart therapeutic efficacy in melanoma, esophageal, and breast cancer orthotopic tumor models.[96,97] FusOn-H2 exhibited no toxicity after i.v. injection in BALB/c mice at a dose 3-logs higher than the wild-type lethal dose, but one out of five mice died after intracerebral injection at 10^7 pfu.[98] HSV2ΔPK was safe after foodpad injection and in a clinical vaccine trial where three injections of 2×10^5 pfu were administered subcutaneously.[99,100]

HSV-2 Us3 has many of the same functions as HSV-1 Us3, including preventing apoptosis, but lacks some of the HSV protein kinase substrates. Thus, HSV-2 Us3 deletions are only minimally attenuated for neurovirulence compared to HSV-1 Us3 deletions.[101] HSV-2 L1BR1 was safe when injected into mouse footpads, but not for intracranial or corneal injections, and induces apoptosis in neurons.[102] In SW1990 pancreatic tumor implanted mice, L1BR1 was significantly better than oHSVs R3616 and hrR3 in inhibiting tumor growth.[77]

Bovine herpesvirus-1

BHV-1, an alphaherpesvirus that affects cattle, causes fever and respiratory disease. BHV-1 has a very restricted host range, likely regulated by IFN. This suggested that it might be inherently oncolytic, like myxoma virus, killing human tumor cells and not normal human cells.[103] The majority of the NCI panel of established human tumor cell lines are permissive to BHV-1 infection and cytotoxicity, with mutations in KRAS associated with virus replication.[104] Direct exposure to BHV-1 seems to be sufficient to induce cell death in many cancer cells, even when replication does not occur.[104]

Equine herpesvirus type 1

EHV-1 is an alphaherpesvirus mainly associated with equine disease that does not infect humans, but does infect human cells in culture. It uses the equine MHCI protein as its entry receptor, which has regions highly conserved across species, including humans.[105] An attenuated EHV-1, deleted for glycoproteins gI and gE (L11ΔgIΔgE), was found to replicate in and kill a number of human glioma cell lines.[105] Given that many tumors, and especially glioblastoma, often

downregulate the expression of MHC 1, it is unclear whether this oncolytic herpes virus is capable of delivering an *in vivo* response.

TRANSCRIPTIONAL AND MICRO-RNA TARGETING

Understanding the lifecycle of HSV-1 allows researchers to target tumor cell populations by altering expression of viral genes to control productive infection in select cell types. ICP4, one of five IE genes, is an essential gene and the major virus trans-activator responsible for activating transcription of the E and L genes.[2] Because ICP4 is essential, regulating its expression with cell-specific transcriptional regulatory sequences can restrict virus replication and oncolytic activity to cells expressing those transcription factors.[90] Thus, oHSV can be targeted to cancer cells based on transcription factor expression in those cells. Alternatively, ICP4 expression can be silenced in normal cells by cell-specific miRNAs targeting ICP4 mRNA containing miRNA target sequences.[106]

Several oHSVs have been constructed with their endogenous ICP4 genes deleted and an ICP4 coding sequence controlled by a cell- or tissue-specific promoter inserted, which confers specificity to a tumor tissue.[90,107–109] These are transcriptionally-targeted oHSVs because they are only able to replicate in cells where expression of a tumor-specific transcription factor drives ICP4 transcription. G92A is the prototypic vector, targeting liver cancer via insertion of ICP4 under control of the albumin promoter into the TK locus.[90] G92A replicates in albumin-expressing Hep3B hepatoma cells, but not the albumin-negative HT29 colon carcinoma cells. In nude mice, G92A completely inhibited the growth of Hep3B, but not prostrate PC3, tumors.[110] *D*12.CALP was similarly constructed, but contains the calponin promoter driving ICP4.[108] Calponin is expressed in many postmitotic cells, but overexpressed in several sarcomas. Thus, *d*12.CALP was efficacious against human leiomyosarcama, but not calponin-negative osteosarcoma xenografts.[108] The Wnt/β-catenin pathway is upregulated in the majority of colorectal cancers and many cancer stem cells, and leads to transcriptional activation of Wnt response elements containing Tcf binding sites.[107] In bM24-TE, a synthetic enhancer containing multiple Tcf binding sites, a 4F2 enhancer, and a CMV minimal promoter was used to drive ICP4 expression after insertion into the ICP6 locus.[107] BM24-TE selectively replicated in and killed cancer cells with strong β-catenin/Tcf signaling (APC or β-catenin mutants), but not cancer cells with low signaling, with similar inhibition of tumor growth.[107]

There have also been several unsuccessful attempts to use cellular promoters to target oHSVs to tumors. Human carcinoembryonic antigen (CEA) is overexpressed in several epithelial tumors. CEAICP4 was created using the CEA promoter to drive ICP4.[111] Unfortunately, it replicated poorly in CEA-expressing cells and not at all in some high-expressing tumors cells,[111] demonstrating that not all tumor associated promoters are good candidates for creating transcriptionally-targeted oHSV. Another problem with transcriptionally targeted oHSV is that the promoter may not behave as expected in HSV. For example, HIF-V6R-HSV, with a HIF-1α-responsive promoter driving ICP4, was active under both normoxic and hypoxic conditions.[112] Confounding the strategy, it was found that HSV-1 infection increased transcription from the HIF responsive promoter in normoxia. In these studies, HIF-V6R-HSV was also found to have reverted back to the wt ICP4 configuration, likely through recombination in complementing cells during its isolation.[112]

Rather than targeting expression of ICP4, a number of oHSV employ regulated expression of γ34.5 to enhance virus replication in tumor cells. This strategy was initially described with Myb34.5, which lacks ICP6 and expresses γ34.5 under a cellular E2F-responsive B-*myb* promoter inserted into ICP6 in γ34.5Δ MGH1 (similar construct to G207).[113] This transcriptional targeting increased the growth of Myb34.5 in human colon cancer cells, but not hepatocytes, and extended survival of mice with liver metastases compared to parental MGH1.[114] The nestin enhancer/Hsp78 minimal promoter drives γ34.5 expression in rQnestin34.5 and 34.5ENVE,[48,115] in order to selectively target tumor cells overexpressing nestin, such as glioblastoma, some peripheral tumors, and cancer stem-like cells.[116] Unfortunately, nestin is also expressed in neural stem/progenitor cells and other tissue stem cells, creating potential safety concerns for nestin-targeted oHSV.[116] RQnestin34.5 and 34.5ENVE replicate well in glioblastoma cell lines and confer a survival benefit in immune-deficient mice implanted intracerebrally with glioblastoma cell lines.[48,117] 34.5ENVE was also effective against patient ascites-derived ovarian cancer cells, which have increased nestin expression.[115] Musashi1 is another gene selectively expressed in glioblastoma. Its promoter was used to drive γ34.5 expression after recombination in G207 to create KeM34.5.[118] KeM34.5 replicated much better in glioma cells, but not musashi1-negative lung or colon cancer cells, than G207, and not in astrocytes.[118] Midkine, upregulated in a number of tumors was used in place of the nestin enhancer to drive γ34.5 expression in oHSV-MDK-34.5 in MPNST.[119]

Several researchers have tested a different method for targeting expression of oHSV genes, in this case silencing expression with microRNAs. MicroRNAs (miR) are small 22-28nt RNAs that signal the destruction of complementary mRNAs via the RISC complex. There are many miRNAs expressed in normal but not tumor cells, so miRNA target sequences inserted into essential HSV-1 genes can selectively inhibit virus replication and/or gene expression in normal cells, leading to selective replication in tumor cells. For example, miR-143 and -145 are expressed in most human tissues but not in prostate cancer cells. MiR-143 and -145 target sequences inserted into the 3′UTR of ICP4 (142T and 145T) blocked its expression in normal but not prostrate cancer cells.[106] The replication of CMV-ICP4-143T, an amplicon plasmid with ICP4Δ helper HSV, in normal tissues was much more restricted than CMV-ICP4-145T.[106] KG4:T124, containing tandem copies of a miR-124 target sequence within the 3′UTR of ICP4, was safe after intracranial injection compared to parent oHSV KG, deleted for the internal repeat region.[120] ICP4-T124 was recombined into KGE, with an EGFR-targeted gD fusion (see Receptor Targeting), and found to be similarly effective in treating mice harboring intracranial glioblastoma stem cell GBM30 tumors as KGE.[120] In LCSOV, miR targets to miR-122a, mir-124a, and miR-let-7a, were used to repress liver-specific apoE-AAT promoter driven gH expression in liver tissue, but not hepatocellular carcinoma.[121] LCSOV was able to replicate in miR122-negative Hep3B cells and inhibit tumor growth.[121]

RECEPTOR TARGETING

The HSV glycoproteins stud the envelope and are responsible for binding to viral attachment factors; heparan sulfate, HVEM (HveA), and nectin-1 (HveC).[122] Upon binding their attachment factors, the glycoproteins aid fusion of the HSV envelope with the plasma membrane. It is possible to modify one or more of the glycoproteins to create a virus that infects cells expressing a targeted receptor and/or detargets cells expressing the normal HSV-1 attachment factors. Glycoprotein C has been used as a site to insert tumor-specific receptor binding ligands or single-chain antibodies (scFv) to retarget virus, but this only removes the heparan sulfate binding

domain.[123,124] Glycoprotein D (gD) binds to the major HSV cell surface receptors, and thus deleting gD binding domains (N-terminus (aa7-32) for HVEM and Ig-core (aa61-218) for nectin 1) and introducing ligands for tumor-specific receptors is an attractive strategy to retarget HSV. Two basic strategies have been used: insert a tumor-specific receptor ligand or scFv into a deletion of a gD-binding domain or detargeted gD mutant[125–127]; or use a recombinant bispecific adapter protein containing the gD-binding domain of nectin 1 or HVEM fused to a tumor-specific receptor scFv with a HVEM or nectin 1 detargeted gD mutant, respectively.[128] HER2 is overexpressed in a number of tumors, including breast and ovarian cancer, and glioblastoma. A HER2 targeted oHSV (R-LM249) was constructed by inserting scFv human HER2 (trastuzumab) into a gD Ig-folded core deletion.[126] R-LM249 inhibited the growth of HER2$^+$ ovarian and breast subcutaneous or metastatic tumors, but not HER2$^-$ SJ-Rh4 rabdomyosarcoma tumors after intraturmoral or intraperitoneal injection.[126,129] A similar HER2-targeted oHSV, R-LM113, replicated in and was efficacious against a PDGF-induced mouse glioma overexpressing HER2 in both immunodeficient and immunocompetent mice, and was safe after intracerebral injection.[130,131] An important caveat to this approach is that HER2 scFv is human specific and does not bind mouse HER2. The use of scFv allows one to potentially target any cell surface molecule, as illustrated by other receptor-targeted oHSVs binding to human EGFR (KNE) or CEA (KNC),[127] or IL13Rα2 (R5141), upregulated in glioblastoma.[125] A confounding feature of this strategy is that all cancer cells must express high levels of the targeted receptor, while cancers are typically heterogeneous for receptor expression.

GENETIC ENGINEERING OF oHSVS

Many researchers now use bacterial artificial chromosome (BAC) systems to quickly swap in transgenes or create specific mutations more easily than classical homologous recombination in tissue culture.[126,132–136] HSV BACs are large circular plasmids (>150 kb), which replicate in bacteria as an artificial genome. BACs are useful for cloning DNAs much larger than is possible using conventional plasmids. For example, the pM24-BAC contains the entire HSV genome with a bacterial origin of replication flanked by Cre/loxP and Flp/Frt sites inserted into the ICP6 gene.[134] To create a new virus, a shuttle vector containing the transgene flanked by Cre/LoxP sites and a stuffer sequence flanked by a Frt/Flp site is recombined into the HSV-BAC with Cre recombinase. The recombined HSV-BAC plasmid is harvested from bacteria and cotransfected into mammalian cells with a Flp recombinase plasmid to remove the BAC and stuffer sequence, and infectious recombinant virus harvested.[134] This method results in a high frequency of recombinant virus. HSV-BACs can also be used to introduce specific mutations in genes, for example through the use of lambda phage Red recombinase.[137]

Aside from BACs, the very recent development of the CRISPR/Cas mutagenesis system allows researchers to quickly insert or remove genes/sequences into the HSV genome without cloning into BAC plasmids, decreasing the time it takes to create a new HSV.[138,139] The CRISPR/Cas9 system is a defense platform for bacteria to target the DNA genomes of bacteriophages. Cas9 is a bacterial nuclease complex, which recognizes a single-stranded guide RNA complementary to a region of dsDNA. The Cas9 enzyme uses the guide RNA to cleave complementary DNA. However, the accuracy of this cleavage is not perfect and mismatches of the guide RNA sequence with the targeted DNA can result in aberrant DNA cleavage. Recently, the CRISPR/Cas9 system was used to insert green fluorescent protein (GFP) into HSV-1, and to introduce gene-specific mutations or repair mutations.[138,139] The CRISPR/Cas9 system is rapidly evolving beyond mutagenesis to gene regulation, with nuclease-deficient Cas9 fused to functional effectors such as transcriptional activators/repressors. CRISPR/Cas9 could also be used to direct proteins to DNA sequences within cancer cells to disrupt oncogenic super-enhancers, epigenetic modifications, or transcriptional complexes.

oHSV-1 EXPRESSING REPORTER OR THERAPEUTIC GENES

Transgenes come in several flavors for creating vectors. Genes are added to visualize the virus' spread, make the virus more cytotoxic to a specific tumor cell population, elicit a targeted immune response, and/or modify the tumor microenvironment. To this end, researchers have outfitted multiple oHSV vectors with imaginative genes to create better vectors. Vectors which express cytotoxic or immune-stimulatory transgenes are usually termed "armed" oHSV,[135] and in the following, we will discuss some of the genes and control mechanisms inserted into several armed vectors.

Reporter genes

The *Escherichia coli* LacZ gene encodes β-galactosidase (β-gal), which cleaves β-bonded sugars, like lactose, into single moieties. A chromogenic substrate of β-gal, termed X-gal, turns blue upon cleavage of its β-bonded sugar, allowing researchers to visualize cells expressing β-gal. LacZ can be inserted into oHSVs in order to monitor their infection of cells *in vitro* and *in vivo*.[22] Several oHSVs have LacZ inserted into the ICP6 gene, derived from hrR3, in order to disrupt ICP6 gene activity and allow for easy monitoring of viral spread (*i.e.*, G207, G47Δ, MG18L (Table 1)).[22,24,25,55] Bioluminescence imaging of luciferase is a noninvasive method for observing viral spread *in vivo*.[119,140–142] If the Luc gene is expressed under a HSV-1 late promoter (*i.e.*, G47ΔUs11fluc), it is possible to monitor the replication of the virus *in vivo* over time, without having to sacrifice the animal.[140,142] GFP expression, for example in NV1066, can also be imaged *in vivo* using fluorescent endoscopy.[143,144] The sodium iodide symporter (NIS) can be monitored noninvasively by CT/SPECT after infected cell uptake of ^{99m}TcO4.[145] In addition, oHSV-NIS can be combined with cytotoxic ^{131}I radiotherapy to enhance antitumor efficacy.[145] The ability to monitor virus biodistribution and replication in live animals and potentially patients provides an important avenue to follow oHSV therapy.

Cytotoxic transgenes

Theoretically, the most straightforward way to increase the efficacy of an oHSV is to arm it with a transgene that is toxic to nearby noninfected tumor cells, in addition to the virus' own cytotoxicity. Expression of prodrug-activating enzymes, suicide gene therapy, is the most common strategy. HSV-TK is the prototypic "suicide gene", converting GCV to GCV-monophosphate, however the toxic metabolite also blocks HSV replication, thereby ablating further virus spread.[5,146] Cytosine deaminase (CD), not found in humans, converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a highly toxic pyrimidine antimetabolite chemotherapeutic. HSV-1yCD and M012 express yeast and bacterial CD, respectively, and the combination with 5-FC significantly increased tumor cell killing, including of noninfected cells (bystander effect), and tumor growth, but not with CD$^-$ parental viruses.[147,148] Other "suicide genes" successfully utilized in oHSV include: rat cytochrome P450 (CYP2B1) in rRp450 and MGH2, converting cyclophosphamide (CPA) into toxic phosphoramide mustard[149,150]; secreted intestinal carboxylesterase (CE) in MGH2, for the conversion of irinotecan (CPT-11) to SN-38[150];

bacterial nitroreductase (NTR) in HSV1790, for the conversion of BC1954 to DNA crosslinker 4-hydroxylamine[151]; and *E. coli* purine nucleoside phosphorylase (PNP) in HSV-PNP, for the conversion of 6-methypurine-2′-deoxyriboside to 6-methylpurine.[152] It is important to bear in mind that some of the toxic metabolites, such as 5-FU and SN-38, can also inhibit HSV replication.[153,154] MGH2 contains two suicide genes, CYP2B1 and CE, and the combination of MGH2 + CPA + CPT-11 was much more effective than MGH2 with either prodrug alone, in an intracerbral glioma model.[150] Another cytotoxic strategy is to express secreted TRAIL, which induces apoptosis after binding to death receptor complexes that are often upregulated in tumor cells. oHSV-TRAIL extended the survival of mice bearing TRAIL- and oHSV-resistant intracerebral gliomas, and was associated with greatly increased apoptosis.[155]

Immune stimulatory transgenes

Although a lively debate is still ongoing, more and more data suggests that a driving force behind oncolytic virotherapy's success is due to immune responses elicited against the tumor.[156] Whether stimulation of the immune system benefits or hinders oHSV therapy is debated due to the efficacy observed in immunodeficient models, which have no adaptive immune system, and the deleterious effects of the innate immune system.[157,158] However, it is clear that robust antitumor adaptive immune responses are elicited by oHSV, as well as protective immune memory, suggesting that activation of the immune system is ultimately responsible for destroying the tumor and preventing its reoccurrence.[81,159,160] Currently, there are several oHSVs that take advantage of stimulating the immune system by expressing immune-stimulatory factors to create a robust T-cell-mediated clearance of the tumor.

Granulocyte macrophage colony stimulating factor has been cloned into several oHSVs (NV1034 and OncoVEX^{GMCSF}).[56,87] Granulocyte macrophage colony stimulating factor stimulates myeloid lineage precursor cells to differentiate, as well as recruiting and activating macrophages and dendritic cells. In immunocompetent mouse models, the expression of granulocyte macrophage colony stimulating factor had only small or no enhanced efficacy over nonarmed oHSV.[56,161,162] OncoVEX^{GMCSF} or currently named Talimogene laherparepvec (T-Vec), the first "armed" oHSV to enter clinical trial, has demonstrated promising efficacy in patients with metastatic melanoma after intratumoral injection, with induction of local and systemic melanoma-specific T cells.[163] In the pivotal phase III OPTiM trial, T-Vec-treated patients had a significant improvement in the durable response rate (16 versus 2% for controls) and a trend toward improved overall median survival.[164]

Using the Flip-Flop HSV-BAC system, a number of immune modulatory transgenes have been inserted into G47Δ (IL-12, IL18, soluble B7.1, and Flt3L), including multiple transgenes in G47Δ-IL18/B7.[133,135,140] IL-12, a heterodimeric cytokine, acts directly to enhance Th1 and cytotoxic T-cell responses, and stimulates IFNγ, with its associated immune effects.[165] IL-12 has been one of the most effective antitumor cytokines expressed from oHSV; M002 in brain tumors,[166] NV1042 in squamous cell carcinoma and metastatic prostate cancer,[87,167] and G47Δ-mIL12 in glioblastoma and malignant peripheral nerve sheath tumors.[140,168] With this success, an oHSV expressing human IL-12 has been constructed, M032, that was safe after intracranial injection in nonhuman primates[169] and is currently in a phase 1 clinical trial for recurrent glioblastoma (http://clinicaltrials.gov/show/NCT02062827). IL-12 also has antiangiogenic activity, which enhances oHSV efficacy in human glioblastoma models in immunodeficient mice.[170] In contrast, treatment of intracerebral gliomas with oHSV expressing immunosuppressive IL-10 (R8308) was no better than mock treatment.[171] In order to prime the adaptive immune response, xenogeneic tumor antigens can be expressed in the tumor, such as prostatic acid phosphatase (PAP) in bPΔ6-hPAP for prostate cancer.[136]

Tumor microenvironment modifying transgenes

Many tumors create a special environment allowing them to grow faster and invade nearby tissues. Growing tumors require new blood vessel formation, angiogenesis, to supply their growing peripheries with nutrients and oxygen. These new vessels also allow infiltration of tumor-associated macrophages, which benefit tumor growth.[172] Neovascularization is promoted by secretion of vascular endothelial growth factor (VEGF) and other angiogenic factors. G47Δ-mAngio encodes the antiangiogenic peptide angiostatin inserted into the ICP6 locus.[173] Infection with G47Δ-mAngio reduced angiogenesis in glioblastoma stem cell and orthopic tumor models, as well as conferring a survival advantage over the empty control virus.[170] The VAE virus is very similar, but expresses a fusion endostatin-angiostatin peptide to dampen angiogenesis.[174] These viruses work under the principle that inhibiting angiogenesis will prevent tumor growth by blocking new blood vessel formation. Other oHSVs expressing antiangiogenic factors include platelet factor-4, G47Δ-PF4, to inhibit endothelial cell growth and migration[175]; Vstat120, extracellular fragment of brain-specific angiogenesis inhibitor 1, expressed by RAMBO and 34.5ENVE[117,176]; thrombospondin-1, a naturally occurring antiangiogenic factor and matrix metalloproteinase (MMP)-9 inhibitor, by T-TSP-1[177]; and endostatin, by HSV-endo.[178]

The tumor extracellular matrix poses a problem for oHSV therapy. Many tumors create an extensive extracellular matrix that limits cell-to-cell interactions within a tumor, and thus cell-to-cell spread of oHSV. MMPs chew through this environment, which opens space for virus transfer. However, MMPs are known to increase the proliferation of many tumors and often lead to metastasis. To avoid this, OV-Chase expresses the bacterial chondroitinase-ABC, which increases interstitial diffusion while maintaining the structural composition of the extracellular matrix.[179] OV-Chase was shown to spread better in glioblastoma spheroids *in vitro*, and increased the survival over nonchondroitinase-ABC expressing rHSVQ.[179] In a reverse approach, rQT3 virus expresses an inhibitor of matrix metalloproteinases, tissue inhibitor of metalloproteinases 3 (TIMP3).[180] rQT3 inhibited the growth of MPNST tumors in mice and MMP activity, and reduced vascular density.[180]

CONCLUSION

There has been an explosion of research on oHSV in the last 25 years, since genetically-engineered HSV was first tested as an oncolytic virus.[6] During that time, eight different oHSVs and "armed" oHSVs have entered clinical trial (G207, 1716, NV1020, OncoVEX^{GMCSF}, HF-10, G47Δ, rRp450, M032 (Table 1)), and OncoVEX^{GMCSF} or T-Vec has successfully completed a pivotal phase 3 clinical trial for recurrent melanoma. Host/tumor interactions that limit virus replication and activity, be they intrinsic cellular responses, innate, or adaptive immunity, will need to be better understood in order to develop strategies to improve efficacy. The potential for developing new oHSV vectors is great, as well as strategies for combining oHSV with other therapeutic approaches to maximize outcomes.[181] The next big step in the field will likely come from more in depth use of clinical tumor isolates to discover patterns and biomarkers associated with certain cancers. Identifying biomarkers, which distinguish cancer subtypes from one another, will allow for the development

of more targeted oHSV, either by transcriptional or receptor targeting. Advances in basic tumor biology to determine what makes a tumor cell or tumor tissue different from normal tissues will allow the creation of oHSV that act like molecular scalpels, infecting and removing only the tumorigenic tissues. When designing or using an oHSV, it is important to closely study host:virus interactions in order to discover new peculiarities within the tumor that can be exploited by the next generation of vectors. Now that CRISPR/Cas9 and BACs are reliably available, it is faster and easier to create new vectors than ever before. Understanding the cellular factors that antagonize virus replication and spread throughout the tumor, as well as contribute to oncolytic activity will create opportunities to improve oHSV therapy, while the clinical trial results will provide insights into the limitations of oHSV and illuminate pathways to success.

CONFLICT IF INTEREST

SDR is a named inventor on patents relating to oncolytic herpes simplex viruses, which were filed by Georgetown University and Massachusetts General Hospital.

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