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Herpes Simplex Virus Type 1 for Use in Cancer Gene Therapy: Looking Backward to Move Forward

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1. Introduction

Herpes simplex virus type 1 (HSV-1) belongs to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily. HSV-1 is a human pathogen associated with keratitis and cold sores, the formation of which is exacerbated by periods of elevated stress, corticosteroid use and immunosuppression. HSV-1 is an enveloped double stranded DNA (dsDNA) virus which following primary infection in epithelial cells follows a retrograde course to establish latency in sensory nerves of the sacral ganglia. Upon reactivation, HSV-1 travels anterograde from latently infected neurons to epithelial cells at the site of primary infection. Antiherpetic drugs such as Acyclovir (ACV) and Gancyclovir (GCV) are used to control herpes virus infection by inhibiting virus replication.

The mature HSV-1 virion consists of an envelope containing 13 different glycoproteins embedded in a lipid bilayer. An icosahedral nucleocapsid encases the dsDNA genome and consists of three different proteins; VP5 forms both hexameric and pentameric complexes that are supported by VP19C/VP23 trimers. The tegument lies between the envelope and capsid and contains viral and cellular proteins, some of which are essential for virus replication. Following cellular entry, capsids dock at the nuclear pore and the genome exits the capsid via channels, which are controlled by tegument proteins.

The HSV-1 genome is ~152 kbp and consists of long and short regions which are covalently linked (Figure 1). Each region is comprised of unique and repeated sequences. Terminal repeat sequences with single nucleotide overhangs allow for circularization during replication and latency. Homologous recombination between these regions produces four isomers in equimolar concentrations (Shen & Nemunaitis, 2006). Overall, the HSV-1 genome codes for approximately 90 proteins, some of which are dispensable for virus replication *in vitro* and some of which are present in more than once copy, making HSV-1 diploid for genes such as infected cell protein 0 (ICP0), ICP4 and ICP34.5.

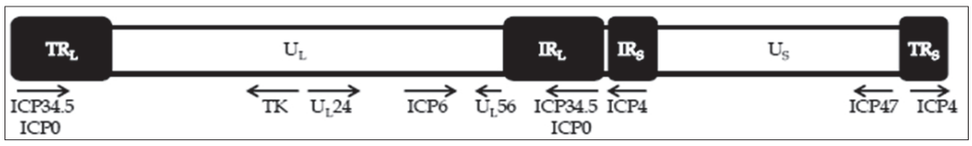

Fig. 1. Schematic of the HSV Genome (TR_L: terminal repeat long, U_L: unique long, IR_L: internal repeat long, IR_S: internal repeat short, U_S: unique short, TR_S: terminal repeat short)

1.1 Cellular entry

Initial attachment of HSV-1 involves binding of glycoprotein B (gB) and/or gC to heparin sulphate proteoglycans on the cellular surface in a low-affinity interaction. This interaction induces a conformational change in gD, relieving steric hindrance of its N-terminal domain and exposing a flexible hairpin loop which binds Herpesvirus entrance mediators (HVEMs) in a high-affinity interaction (Heldwein & Krummenacher, 2008). Members of the immunoglobulin superfamily, nectin-1 and nectin-2, bind via a separate site at tyrosine 28 of gD (Heldwein & Krummenacher, 2008). The binding sites of HVEM and nectin do not overlap and thus require different conformations of gD for binding to occur. Downstream signalling cascades induce a conformational change in gB and/or gC and the gH/gL complex to bring about full fusion of the virus envelope with the cellular membrane. The viral capsid is released into the cytoplasm, and along with several tegument proteins, travels to the nucleus via the microtubular network (Shen & Nemunaitis, 2006). The HSV-1 genome enters the nucleus via nuclear pores to initiate virus replication. While HSV-1 has also been shown to enter cells via endocytosis, the factor(s) which dictate this method of entry remain uncertain.

1.2 Virus replication

Upon entry into the nucleus the HSV-1 genome is transcribed in a highly regulated cascade proceeding from immediate early (IE) genes, to early (E), then late (L) genes. Each gene functions as an independent transcriptional unit through a separate promoter. In general, genes are clustered based on function, not on the order in which they are transcribed. For a comprehensive review, please refer to Roizman & Sears (1996).

Transcription is initiated through association of VP16, a L gene product which resides in the tegument, with the cellular DNA-binding protein Oct-1, which binds the "TATGARAT box" within the enhancer region of IE promoters. The C-terminus of VP16 is a potent transcriptional activating domain which enhances RNA polymerase II (RNA Pol II) activity. There are five IE genes, ICP0, ICP4, ICP22, ICP27 and ICP47, four of which have functions as regulatory factors to initiate lytic infection, while the fifth, ICP47, functions in immune evasion. ICP4 is a major transcriptional activator essential to viral replication. ICP0 acts along with ICP4 to enhance E gene transcription and is also involved in countering the host antiviral response. In addition, ICP22 and ICP27 enhance viral gene transcription by regulating activation of RNA Pol II and modulating post-transcriptional events, respectively. When sufficient levels of IE genes have been transcribed, ICP4 inhibits IE gene expression and activates E gene expression. This switch is mediated by the activation of host RNA Pol II by IE gene products and results in the expression of proteins essential for viral replication such as DNA polymerase, single-strand DNA binding proteins, helicase-primase complex and ori-binding proteins. The accumulation of E gene products results in viral DNA replication at replication compartments located in the nucleus. Lastly, L genes are transcribed following DNA replication and code for structural proteins such as those included in the nucleocapsid and viral envelope. Rolling circle replication produces concatemers which are cleaved by pac1/2 enzymes at specific sequences forming monomers which are then packaged into a nucleocapsid. At this time several tegument proteins associate with the newly formed nucleocapsid and help direct budding through the inner nuclear membrane, resulting in acquisition of an immature envelope. The immature envelope is lost and a new one is formed as the virion fuses and passes through the outer nuclear membrane into the

cytoplasm. Additional tegument proteins, such as VP16, associate with the nucleocapsid to direct formation of the mature envelope, which is acquired as the virion passes through the Golgi complex to the cellular membrane. Incorporation of glycoproteins into the envelope occurs at the Golgi and functions to direct the virus to the cellular membrane from which the new virion will bud.

1.3 Latency

HSV-1 is a neurotropic virus which establishes life-long latency within the neurons of its host. Primary infection occurs in epithelial cells after which the nucleocapsid and tegument are transported retrograde along sensory neurons at the site of infection. During latency, viral DNA associates with histones to form an episome and active replication does not occur (Shen & Nemunaitis, 2006). However, latency-associated transcripts (LATs) are abundantly transcribed during latency and are antisense (in part) to the gene transcript which encodes ICP0, suggesting it may interfere with ICP0 synthesis to prevent productive gene expression from occurring in latently infected neurons. Furthermore, LATs do not code for protein products but rather are alternatively spliced RNA species forming two different transcripts (2 and 1.5 kb) of varying abundance (Shen & Nemunaitis, 2006). LATs have also been shown to protect neuronal cells from apoptosis by inhibiting both extrinsic and intrinsic apoptosis pathways by blocking caspase 8 and 9 activity, respectively (Ahmed et al., 2002; Henderson et al., 2002). Reactivation from latency occurs following cellular stress, increases in corticosteroid and/or hormone levels and immunosuppression, resulting in limited productive gene expression and viral replication. Newly formed virus travels anterograde to re-infect epithelial cells within the site of primary infection.

1.4 Host defense mechanisms

From the moment HSV-1 infects host cells at the site of primary infection, viral mechanisms are in place to counteract host immune responses. The innate immune response is the first line of defense against HSV-1 infection followed by high-affinity adaptive responses. HSV-1 encodes proteins which function to evade the host interferon (IFN) response (Paladino & Mossman, 2009), elude complement system opsonisation (Wakimoto et al., 2003), inhibit antigen presentation on MHC I molecules (Hill et al., 1995), block synthesis of host proteins (Shen & Nemunaitis, 2006), hinder apoptosis, and impede maturation of dendritic cells (DCs) (Kobelt et al., 2003).

Innate cytokines including IFN are produced in response to HSV-1 infection. There are three types of IFN, type I, II and III, with type I and III comprising multiple species. Type I and III IFN are expressed by many different cell types, however type II IFN is primarily produced by activated T cells and natural killer (NK) cells. The effects of IFN are pleiotropic and include antiproliferative, antiviral and immunoregulatory functions executed through the expression of interferon stimulated genes (ISGs) (Sen & Sarkar, 2007). Overall, IFN signalling serves to expand the repertoire of immune effectors involved in the antiviral response and thereby regulates viral replication and spread. The production of IFN in most cell types occurs in three phases: sensitization, induction and amplification. During the sensitization phase, pathogen-associated molecular patterns are recognized by pattern recognition receptors including members of the toll-like receptor (TLR) and retinoic acid-inducible gene-I receptor families. Recognition results in the activation of several cellular transcription factors, including interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and the subsequent expression of

IFNβ. During the inductive phase, secreted IFNβ functions in a positive feedback mechanism by binding its cognate receptor. This leads to expression of additional ISGs via the Janus-activated kinase and signal transducer and activator of transcription (STAT) pathway. The interferon stimulated gene factor 3 (ISGF3) complex, consisting of STAT1, STAT2 and IRF9, translocates to the nucleus and binds the promoter of ISGs, including IRF7. Once activated, the IRF3-IRF7 heterodimer induces expression of additional ISGs including IFNα species. Lastly, during the amplification phase IFNα/β stimulate the production of ISGs such as dsRNA-dependent protein kinase receptor (PKR) and oligoadenylate synthetase (OAS) pathways, which function to inhibit viral protein synthesis and degrade viral RNA, respectively. Due to the pleiotropic effects of IFN during infection, HSV-1 has evolved mechanisms by which to inhibit this potent antiviral response.

Infected cell protein 0 (ICP0) is an IE gene product which is pivotal in counteracting the IFN response. HSV-1 ICP0-null mutants are hypersensitive to IFN such that production of viral mRNA and resultant protein is diminished, leading to reduced plaque forming ability (Mossman et al., 2000). The diverse mechanisms by which ICP0 counteracts the IFN response include inhibition of IRF3, IRF7, and STAT1 activation, and degradation of promyelocytic leukemia protein (PML) (Paladino & Mossman, 2009; Lin et al., 2004; Halford et al., 2006; Everett et al., 1998; Sobol & Mossman, 2006). Although the E3 ubiquitin ligase activity of the RING finger domain of ICP0 has been implicated in the inhibition of cytoplasmic IRF3, this mechanism has been shown to be proteasome-independent (Paladino et al., 2010). In addition to ICP0, the IE gene ICP27 functions in IFN evasion by repressing host transcription, translation, mRNA splicing and induces mRNA degradation. Through these mechanisms ICP27 inhibits IRF3 and STAT1 activation (Melchjorsen et al., 2006; Lin et al., 2004; Melroe et al., 2004; Johnson et al., 2008). However, it should be noted that the ability of ICP27 to inhibit IRF3 may be cell type specific. Deletion of ICP27 does not result in an increase in ISG production in primary human fibroblasts, while the opposite was observed in human macrophages (Melroe et al., 2004; Melchjorsen et al., 2006).

Furthermore, several L proteins including ICP34.5, Us11, virion host shutoff (vhs) and Us3 inhibit the IFN response. ICP34.5 acts in concert with the IRF3 kinase TBK-1 to inhibit IRF3 activation (Verpooten et al., 2009) and with the cellular phosphatase PP1 to reverse PKR-induced inhibition of viral protein synthesis (Chou et al., 1995; He et al., 1997). Us11 inhibits PKR activation by binding PKR itself, or activators of PKR such as dsRNA and PACT (Peters et al., 2002). Us11-mediated inhibition of PKR has been found to enhance the growth of ICP34.5-null HSV-1 (Poppers et al., 2000). Conversely, PKR activation levels in Us11-null HSV-1 infected cells are reduced in comparison to levels in wild type HSV-1 infected cells (Mulvey et al., 2003). Together these data demonstrate an additive effect of Us11 in PKR inhibition. The ability of Us11 to bind dsRNA at late phases of infection prevents the synthesis of 2′-5′ oligoadenylates by inhibiting OAS activation (Cayley et al., 1984; Sanchez & Mohr, 2007). It has been shown that Us11 and OAS directly interact at RNA-containing complexes and can bind the same RNA molecules (Sanchez & Mohr, 2007). The vhs protein is integral in counteracting the host antiviral response, in particular the type I and II IFN signalling cascades. In addition to its roles in inhibiting host protein synthesis by degrading mRNA and disrupting polyribosomes, vhs regulates the expression of ISG transcripts. A study by Leib et al. (1999) found that vhs-null HSV-1 replication was severely reduced in non-dividing cells *in vitro* as well as *in vivo*, resulting in viral clearance within 24 hours. Moreover, vhs mutants are hypersensitive to IFN (Pasieka et al., 2008) and in comparison to wild type HSV-1 show a marked increase in

ISG accumulation (Lin et al., 2004). vhs has also been shown to induce the degradation of IFNγ-specific transcripts, thereby inhibiting type II IFN signalling. Furthermore, vhs inhibits STAT-1 activation and formation of the ISGF3 complex following IFNγ treatment of human DCs (Eisemann et al., 2007; Chee & Roizman, 2004). Lastly, Us3 functions as a protein kinase to impede apoptosis thereby promoting viral gene expression (Leopardi et al., 1997). With respect to the type I and II IFN response, Us3 alters TLR3-mediated signalling by decreasing TLR3 mRNA expression, resulting in depleted IRF3 homodimerization (Peri et al., 2008). Additionally, Us3 post-translationally modifies type II IFN receptors via phosphorylation, resulting in decreased expression of IFNγ-dependent genes (Liang & Roizman, 2008).

The complement system is activated in response to HSV-1 infection leading to formation of a membrane attack complex and lysis of the infected cell. By-products of the complement system recruit inflammatory cells to the site of infection resulting in opsonisation by macrophages. To evade the complement system, HSV-1 gC binds to and inactivates C3, the central convertase in the complement cascade, as well as other components such as C5 and properdin (Wakimoto et al., 2003). This response is variable due to differences in the binding efficiency of gC to its' targets between different strains of virus (Wakimoto et al., 2003). In addition, activity of virus-specific antibodies has been shown to enhance the antiviral activity of the complement system by aiding in activation of the classical pathway (Wakimoto et al., 2003).

Activation of cellular immune effectors is critical to controlling infection, as well as determining the type and strength of the adaptive immune response. Macrophages, NK, DC, cytokines and chemokines control the initial infection and prevent its spread to other tissues (Wakimoto et al., 2003). Persistence of CD8+ T cells in infected neuronal tissue has been implicated in controlling HSV-1 during latency and reactivation (Knickelbein et al., 2008). DCs are potent antigen presenting cells as they are able to induce naïve T cells which in turn stimulate maturation of B cells resulting in production of HSV-1-specific antibodies. HSV-1 is able to infect DCs and thereby block maturation via the IE protein ICP27 (Kobelt et al., 2003). This results in the inability of DCs to induce a potent immune response and antiviral T cell stimulation. HSV-1 ICP47 blocks transporter associated with antigen processing (TAP) preventing translocation of peptides to the endoplasmic reticulum (ER) and thus MHC I formation, ultimately leading to a decrease in activation of CD8+ T cells. Furthermore, vhs reduces expression of MHC I & II, IL-1β and IL-8 and activation of DCs (Eisemann et al., 2007; Chee & Roizman, 2004; Suzutani et al., 2000). Due to repressed CD8+ T cell activity, CD4+ T cells migrate to the area of infection where they secrete cytokines to activate NK cells. This culminates in secretion of IFNγ which increases MHC I production and thus CD8+ T cell activation.

2. Oncolytic virotherapy

The use of viruses to target and lyse cancer cells is a novel approach to cancer therapy that lacks the toxic side effects of many cancer treatments that are currently used. Furthermore, virotherapy has been used in cases where cancer cells have become apoptosis-resistant and are refractory to common treatment modalities. Although the safety and potential benefit of using viruses has been addressed in phase I and II clinical trials, the efficacy remains poor (Ries & Brandts, 2004). Thus, the search for new cancer therapy approaches with a high therapeutic index but limited pathogenicity continues.

Oncolytic virotherapy is based on the observation that some viruses preferentially replicate in and kill cancer cells while having minimal detrimental effects on normal cells (Cervantes-García et al., 2008). It holds potential as a successful avenue of gene therapy given its two-pronged approach, the destruction of cancer cells as a direct result of viral replication, and induction of tumour-specific immunity (Vaha-Koskela et al., 2007). Oncolytic viruses lack the widespread toxicity of chemotherapeutics with local replication of the virus amplifying the input dose and resulting in spread to adjacent tumor cells.

Oncolytic viruses can be divided into two groups, wild type viruses that are naturally oncolytic and do not require mutations to render them oncotropic and those that require genetic modification for selective oncolysis. HSV-1 was the first virus used to demonstrate that a genetic mutation can render a virus oncolytic (Jia & Zhou, 2005, Shen & Nemunaitis, 2006). In fact, HSV-1 has been studied extensively as an oncolytic virus due to the many advantages it possesses for use in virotherapy. A brief section on natural oncolytic viruses has been included and will be followed by a detailed discussion of genetically modified HSV-1 vectors.

2.1 Natural oncolytic viruses

Wild type viruses including, but not limited to, reovirus, measles virus (MV), Newcastle disease virus (NDV), myxoma virus (MYXV), vesicular stomatitis virus (VSV), and Bovine herpesvirus type 1 (BHV-1) do not require a mutation to selectively replicate in tumor cells. These viruses exploit biochemical differences between normal and tumour cells to allow for preferential replication in cancer cells (Vaha-Koskela et al., 2007). In addition, species-specific viruses have gained popularity for use in oncolytic virotherapy due to the absence of pre-existing immunity to these viruses. This lack of immunity prevents premature neutralization through antibody-mediated opsonisation and may also allow for systemic delivery of the virus, therefore improving the therapeutic potential against metastatic lesions.

The selective replication of oncolytic viruses is the result of a collection of gain- or loss-of-function mutations in a given cancer type (Vaha-Koskela et al., 2007). An example of one essential modification and hallmark of most malignant cancer types is the evasion of apoptosis (Hanahan & Weinberg, 2000). This is often the result of gain-of-function mutations in cellular signalling pathways, such as in constituents of the Ras signalling cascade (Ayllon & Rebollo, 2000). Activating mutations in Ras inhibit autophosphorylation of PKR, an important effector molecule in the IFN-mediated antiviral and apoptotic response (Everts & van der Poel, 2005). Wild type viruses that are sensitive to the effects of PKR, such as reovirus and NDV, can infect and replicate in cells with a Ras activating mutation, such as in cancer cells, while remaining inhibited in normal cells (Strong et al., 1998). In addition to its function as a cell cycle and apoptosis regulatory protein, p53 also functions as a potent tumor suppressor. In fact, p53 is the most commonly mutated oncogene in human cancers with over 50% of tumor cells carrying a mutation in p53 (Morris et al., 2002). Furthermore, p53 amplifies innate immune responses to virus infection through such mechanisms as enhancing IFN-dependent responses and increasing IRF9 gene transcription (Muñoz-Fontela et al., 2008). Therefore, disruption of p53 is thought to dampen antiviral defense mechanisms conferring heightened sensitivity of cells to infection (Carroll, P.E. et al., 1999). Reovirus and MYXV replicate to higher levels in cancer cells with reduced expression of p53, and although this is not the major determinant dictating their selective replication in tumor cells, it enhances efficiency of infection (Kim et al., 2010).

Another feature of cancer cells that is often exploited by oncolytic viruses is an impaired IFN response. Upon binding to cellular receptors, IFN induces the expression of ISGs, many of which function to block viral replication (Platanias, 2005; Everts & van der Poel, 2005). A well characterized ISG involved in blocking viral replication is PKR, as discussed previously (Sadler & Williams, 2008; Everts & van der Poel, 2005). In addition to antiviral functions, many ISGs have been identified as tumour suppressors, including PML, ribonuclease (RNase) L, and ISG15 (Everts & van der Poel, 2005; Bernardi & Pandolfi, 2003; Salomoni & Pandolfi, 2002). Furthermore, IRFs have been shown to possess tumour suppressive functions (Pitha et al., 1998; Taniguchi et al., 2001; Shen & Nemunaitis, 2006). For example, IRF-1 has been implicated in cell cycle regulation and apoptosis (Tamura et al., 2008), and the development of some cancers, such as breast (Connett et al., 2005) and hepatocellular carcinoma (Moriyama et al., 2001), is correlated with decreases or loss of IRF-1 expression. Other pathways involved in IFN production, such as NFκB, have been implicated in tumour suppression (Everts & van der Poel, 2005; Chen & Castranova, 2007). Given the diverse antitumorigenic functions of IFN signalling it is common for tumour cells to harbour defects in these pathways, and oncolytic viruses that are sensitive to IFN, such as VSV and NDV, preferentially replicate in tumor cells over normal cells (Jia & Zhou, 2005; Ries & Brandts, 2004; Everts & van der Poel, 2005).

2.1.1 Reovirus

Reovirus is a non-enveloped dsRNA virus which has minimal pathogenicity in humans but can cause gastrointestinal and respiratory symptoms. Selective replication of reovirus occurs in tumor cells harbouring activating mutations in the Ras pathway (Strong et al., 1998; Coffey et al., 1998), as outlined above. Reovirus has shown promise in pre-clinical studies with selective replication in tumors of breast (Strong et al., 1996; Norman et al., 2002; Hirasawa et al., 2002), colon (Kinzler & Vogelstein, 1996), ovarian (Hirasawa et al., 2002), and lymphoid (Alain et al., 2002) origins. Combination therapy involving the immunosuppressant cyclosporine A or anti-CD4+/CD8+ antibodies enhances the oncolytic ability of reovirus in systemic treatment of metastatic malignancies (Hirasawa et al., 2002). A phase I open-label dose-escalation trial with reovirus type 3 Dearing (RT3D) in combination with radiotherapy was performed on twenty-three patients with advanced or metastatic solid tumors (Harrington et al., 2010a). Patients received intralesional injections of RT3D ($1x10^8$ pfu up to $1x10^{10}$ pfu) and were monitored for tumor regression and adverse events. Treatment was well tolerated with no adverse effects associated with virus treatment. Overall, seven patients achieved partial response and seven achieved stable disease. Results of this and other clinical trials using reovirus for the treatment of malignancies support development of this virus as a therapeutic option.

2.1.2 Measles virus

Measles virus (MV) is an enveloped (-)ssRNA virus that causes rash, fever, cough and conjunctivitis in infected humans. Several cases of spontaneous tumor regression have occurred in individuals with MV infection, suggesting an oncolytic attribute for the virus. However, the cellular receptor SLAM, which is used by wild type MV for cellular entry, is not commonly expressed on tumor cells. A live attenuated measles strain with no pathogenicity in humans has been generated by serially passaging the virus in tissue culture. This virus has adapted to use CD46, an inhibitor of complement activation, for cellular entry (Peng et al., 2003a). The CD46 receptor is expressed in higher abundance on

human tumor cells than on their non-transformed counterparts, rendering the attenuated strain oncolytic. Attenuated MV has been used to treat B cell malignancies such as non-Hodgkin's lymphoma and myeloma (Grote et al., 2001; Peng et al., 2001). In these studies the formation of multinucleated syncytia increased viral spread and thus the efficacy of virus treatment. In addition, a MV expressing an antibody against CD38, a cell marker highly expressed on myeloma cells, induced cell-cell fusion and cytopathic effects in mouse xenografts resulting in inhibition of tumor growth and prolonged survival (Peng et al., 2003b).

2.1.3 Newcastle disease virus

Newcastle disease virus (NDV) is an enveloped (-)ssRNA avian paramyxovirus that is non-pathogenic to humans. Like reovirus, NDV is unable to counter the effects of PKR and thus requires activated Ras to replicate in tumor cells. While Ras is the main factor determining NDV tropism, mutations in the IFN signalling pathway also confer sensitivity to NDV. Live attenuated strains of NDV have shown efficacy in mouse models of fibroblastoma (Lorence et al., 1994a), neuroblastoma (Lorence et al., 1994b; Reichard et al., 1993), colon (Schulze et al., 2009), prostate (Phuangsab et al., 2001), melanoma (Zamarin et al., 2009), large cell lung (Phuangsab et al., 2001) and breast carcinoma (Zhao et al., 2008; Janke et al., 2007). Moreover, live attenuated NDV strains MTH-68/H and PV701 are currently in clinical trials for high-grade gliomas (HGG) and advanced solid cancer, respectively (Csatary et al., 2004; Pecora et al., 2002).

2.1.4 Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is an enveloped (-)ssRNA virus that is non-pathogenic to humans. VSV is exquisitely sensitive to the effects of IFN signalling and as such selectively replicates in tumor cells harbouring mutations in IFN and its signalling effectors (Stojdl et al., 2000). The oncolytic ability of VSV has been demonstrated in *in vivo* models of melanoma (Luo et al., 2010; Galivo et al., 2010), colon, and hepatocellular carcinoma (Shinozaki et al., 2004). In a recent study, recombinant VSV expressing human dopachrome tautomerase (hDCT) was used as an immune boost resulting in enhanced efficacy in the B16-F10 model of melanoma. Results indicate the efficacy of VSV in augmenting the antitumor immune response (Bridle et al., 2009).

2.1.5 Myxoma virus

Myxoma virus (MYXV) is an enveloped dsDNA virus that is non-pathogenic in humans. It causes Myxomatosis in European rabbits, which is uniformly fatal due to immunomodulation by several virus-encoded proteins (Stanford et al., 2007). Even in cases of routine exposure to the virus humans do not seroconvert (Stanford & McFadden, 2007). MYXV is restricted from normal mouse cells due to the IFN response, and from normal human cells due to IFN and tumour necrosis factor (TNF) responses (Lun et al., 2005). MYXV has been shown to selectively replicate in human cancer cells of various histological origins (Sypula et al., 2004; Lun et al., 2005, Wang et al., 2008). The cellular tropism of MYXV for human tumor cells largely depends on the interaction of a viral host range factor (M-T5) and cellular Akt, a protein kinase with roles in cellular proliferation, migration, transcription and apoptosis (Wang et al., 2006). This interaction has been shown to induce the kinase activity of Akt resulting in MYXV replication. In a xenograft model of human

glioma in immunocompromised mice, intracranial injection of MYXV was well-tolerated and caused regression of the injected tumor. Regression in distal tumors was not observed but virus replication was sustained long term (Lun et al., 2005). Combination therapy trials using rapamycin, an immune and tumor suppressant which upregulates Akt activation via the mTOR pathway, resulted in increased MYXV replication in select tumor types (Werden et al., 2010).

2.1.6 Bovine herpesvirus type 1

Bovine herpesvirus type 1 (BHV-1) is a member of the Herpesviridae family, and Alphaherpesviridae subfamily. BHV-1 causes infectious bovine rhinotracheitis (IBR) in cattle, manifesting in symptoms such as ocular and nasal secretions, lesions on mucosal surfaces, anorexia, dyspnoea, conjunctivitis, and abortions (Turin et al., 1999, Hushur et al., 2003). In approximately 10% of affected animals bacterial superinfection occurs, resulting in bronchopneumonia (Turin et al., 1999). However, in the absence of bronchopneumonia the infection is self-limiting due to the immune response, and recovery occurs within 1 to 2 weeks (Turin et al., 1999). BHV-1 is a neurotropic virus which establishes life-long latency in neurons, with reactivation of the virus resulting from parturition, pregnancy, transport, entrance into a new herd, concomitant bacterial or viral infections, poor living conditions, deficient diet and increases in corticosteroids (Turin et al., 1999, Jones & Chowdhury, 2007).

The structure of BHV-1 is similar to that of HSV-1, however some important differences exist. BHV-1 binds attachment and entry receptors used by HSV-1, such as heparan sulfate and nectin-1 (Campadelli-Fiume et al., 2000). However, it is unable to bind nectin-2, but binds CD155 instead (Campadelli-Fiume et al., 2000). CD155 is a poliovirus receptor associated with tumour cell migration and invasion, and has been shown to be up-regulated in human cancers (Merrill et al., 2004; Pende et al., 2005). Genes expressed by BHV-1 are generally named after the coinciding HSV-1 gene, which often have similar activities although there are some functional differences. For example, bICP0 is similar to HSV-1 ICP0 in that it is an IE gene product which acts as a transcriptional activator and possesses E3 ubiquitin ligase activity inherent to the RING-finger domain. However, bICP0 differs from HSV-1 ICP0 in that it promotes proteasomal-dependent degradation of IRF-3 (Henderson et al., 2005, Saira et al., 2007). Furthermore, while ICP0 induces disruption of PML nuclear bodies (PML-NB) by degrading SUMO-modified forms of PML, bICP0 does not decrease steady-state levels of PML but rather degrades sp100, another major component of PML-NBs (Everett et al., 2010).

Of particular interest is the narrow host range of BHV-1, as it is unable to productively infect mice and normal human cells (Hushur et al., 2003). Also, cattle farmers whom are routinely exposed to the virus do not seroconvert. Rodrigues et al. (2010) showed that human transformed and phenotypically normal immortalized cell types are permissive to BHV-1, while normal primary cell types are relatively resistant to BHV-1 infection (Figure 2). BHV-1 induces cytopathic effects (CPE) and decreases cellular metabolism in immortalized and transformed cells from the lung, mammary, bone and prostate origins with varying efficiency.

Naturally occurring oncolytic viruses such as VSV, MYXV and NDV are sensitive to the effects of type I IFN signalling. However, this is not the case for BHV-1 as cellular IFN-responsiveness does not correlate with sensitivity to BHV-1 (Table 1) (Rodriguez et al.,

2010). IFN production was also absent in BHV-1 infected cells. These observations suggest that a broader factor, or group of factors, limit BHV-1 replication in normal cells.

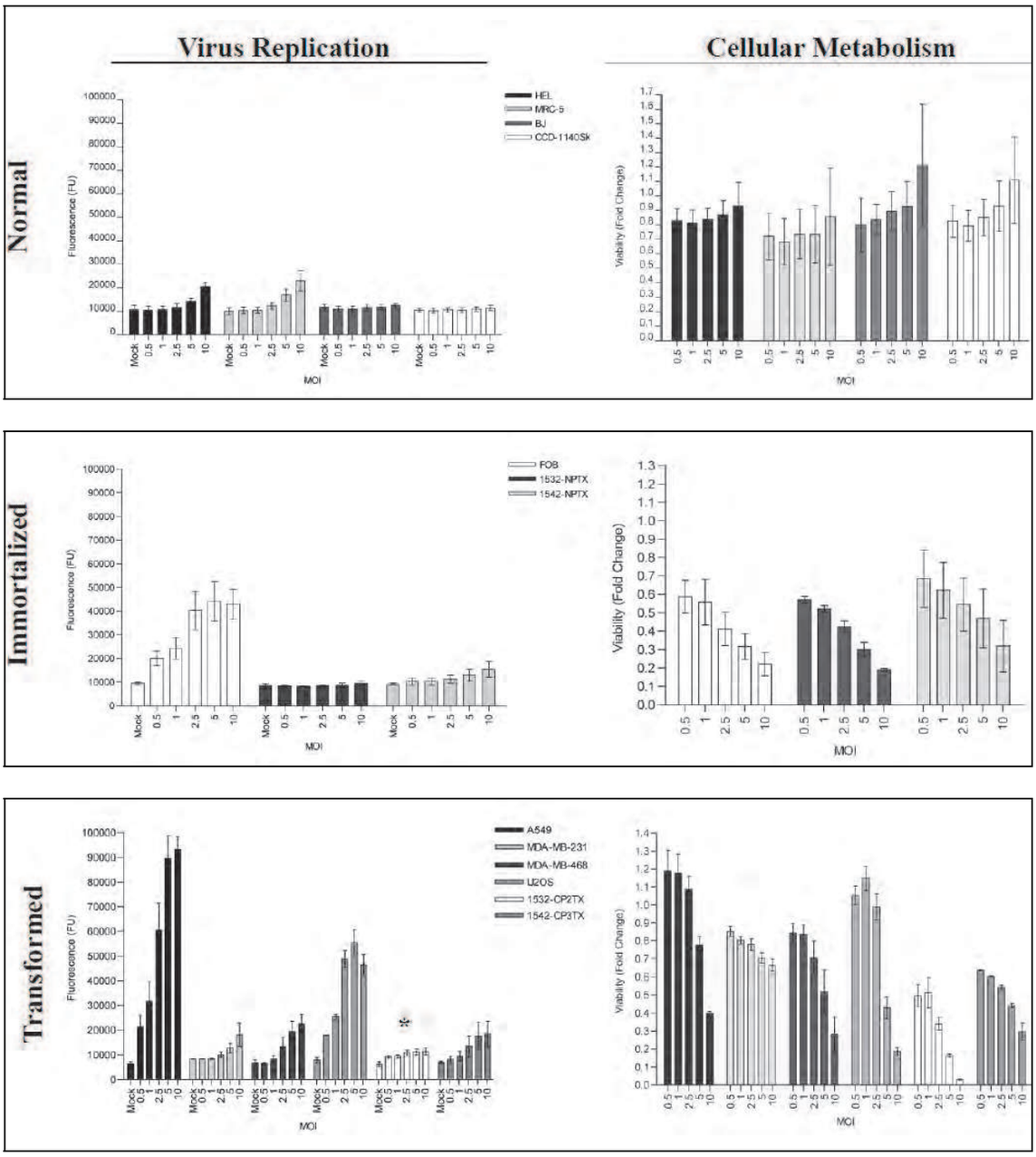

Fig. 2. BHV-1gfp replication and cellular viability in normal, immortalized and transformed human cells. Virus replication, as a function of GFP fluorescence, was quantified two days post infection using a Typhoon Bioanalyzer (Amersham Biosciences). Virus replication is represented in absolute fluorescence units (FU). Cellular metabolism was measured using Alamar blue and is represented as a fold change over uninfected controls. (*) indicates loss of fluorescence due to the absence of viable cells. Data is expressed as the mean from three independent experiments. (Rodriguez et al., 2010)

State	Tissue Type	Cell Line	EC_{50} (U ml^{-1})	CPE
Normal	Lung	HEL	1.499	-
		Ronald	0.538	-
		Ventressca	0.705	-
	Skin	CCD-1140Sk	0.362	-
Immortalized	Mammary	MCF-10A	0.500	++
		HME-1	1.198	+++
	Bone	FOB	1.215	+++
	Prostate	RWPE-1	0.680	+++
		1542-NPTX	0.769	+
Transformed	Lung	A549	3.554	+++
	Mammary	MDA-MB-231	1.317	+
		MDA-MB-468	3.548	+++
	Bone	U2OS	41.249	+++
	Bone metastasis of prostate origin	PC-3	7.226	++
		PC-3M	2.867	++
		PC-3M-M2	10.327	++
		PC-3M-Pro4	8.303	++
		PC-3M-LN4	10.047	+

Table 1. IFN responsiveness for normal, immortalized and transformed cells from a variety of histological origins. Cells were treated with human IFNα for 24 hours prior to infection with VSV-GFP. VSV replication was used to assess the IFN responsiveness of each cell line, as cells responsive to IFN will be unable to support VSV replication. The effective concentration of IFN required to inhibit 50% of GFP fluorescence (EC_{50}) was calculated to compare IFN responsiveness between cell lines. Cytopathic effect (CPE) scoring was performed by infecting cells with BHV-1 at an MOI 0.5, 1. 2.5, 5 or 10. Three days pi cell monolayers were stained with Giemsa to evaluate CPE. (- indicates a non-permissive cell type, no visible CPE; + indicates a cell type that is not very permissive, 50% CPE at MOI >2.5; ++ indicates a moderately permissive cell type, 50% CPE between MOI 1 and 2.5; +++ indicates a highly permissive cell, 50% CPE at MOI <1) (Rodriguez et al., 2010).

These data suggest that BHV-1 holds promise for use in broad spectrum oncolytic virotherapy. The non-pathogenic nature of BHV-1 and lack of pre-existing immunity in humans, as well as the potential for systemic treatment with increased safety offers an advantage over current HSV-1 vectors.

2.2 Engineered oncolytic viruses: HSV-1 vectors

Genetically modified tumor-selective oncolytic viruses are often generated from the deletion of genes that are required for replication in normal cells, but are dispensable in tumor cells. A wide variety of viruses have been engineered for oncolytic virotherapy, including but not limited to, HSV-1, adenovirus, vaccinia virus, poliovirus, and influenza virus. Pathways commonly mutated in tumor cells, such as those involved in regulation of cell cycle and proliferation, are often those exploited by viruses. For example, cell cycle checkpoint

proteins p53 and retinoblastoma (Rb), and the Ras family of proteins and their downstream effectors, are commonly mutated in solid and hematologic cancers (Ayllon & Rebollo, 2000). Thus, oncolytic viruses have been generated to selectively replicate in tumor cells possessing these and other mutations. This section will focus on discussion of engineered HSV-1 oncolytic viruses.

2.2.1 First generation vectors

HSV-1 was the first virus used to demonstrate that a genetic mutation can render a virus oncolytic (Jia & Zhou, 2005; Shen & Nemunaitis, 2006). In fact, HSV-1 has been studied extensively as an oncolytic virus due to the many advantages it possesses for use in virotherapy. Its large dsDNA genome (~150 kbp) allows for the insertion of multiple transgenes, up to 30 kbp in length (Everts & van der Poel, 2005; Shen & Nemunaitis, 2006). HSV-1 has been shown to infect a broad number of cell types due to the ubiquitous expression of its receptor in human tissues. The virus is able to kill infected cells as a result of lytic replication and does not insert its DNA into the host genome, reducing the risk of insertional mutagenesis (Varghese & Rabkin, 2002; Shen & Nemunaitis, 2006). Moreover, HSV-1 rarely causes severe illness in immunocompetent adults (Varghese & Rabkin, 2002; Shen & Nemunaitis, 2006) and antiherpetic drugs, such as ACV and GCV, can be used to control viral replication.

Common mutations in HSV-1 oncolytic viruses are in the genes that encode for thymidine kinase (tk) and ribonucleotide reductase, also known as ICP6. These genes are responsible for synthesis of deoxynucleotide triphosphates (dNTPs) and are required for virus replication. Cellular synthesis of dNTPs is upregulated during G1 and S phases of the cell cycle but otherwise remain low. Thus, mutation of the genes encoding tk and ICP6 restrict replication to rapidly proliferating tumor cells. However, studies of the tk-null mutant dlsptk (Table 1) in severe combined immunodeficient (SCID) mice indicated the spread of the virus to normal tissue (Valyi-Nagy et al., 1994). Presence of an intact tk gene can serve as a safe guard against unwanted replication through the use of antiherpetic drugs, such as ACV and GCV. The HSV-1 recombinant hrR3 (Table 1), which is deleted for ICP6, selectively replicates in tumor cells with active ribonucleotide reductase due to their high rate of proliferation. ICP6-null mutants retain sensitivity to ACV and GCV, adding to their safety profile. This mutant has shown promising antitumor effects in animal models of brain (Mineta et al., 1994), pancreas (Kasuya et al., 1999), colon (Yoon et al., 2000; Carroll, N.M. et al., 1996), and liver (Pawlik et al., 2000) carcinoma. In combination therapy trials, co-administration of cyclophosphamide (CPA) or cobra venom factor (CVF) and hrR3 resulted in enhanced antineoplastic effects (Ikeda et al., 2000).

The leaky L gene ICP34.5 is a neurovirulence factor, which when deleted inhibits virus replication in neurons and other cells with a slow doubling time. ICP34.5 also functions to reverse PKR-mediated inhibition of viral protein synthesis. A wide variety of ICP34.5-null HSV-1 mutants have been described which preferentially replicate in cancer cells with Ras gain-of-function mutations (Davis & Fang, 2005; Farassati et al., 2001; Shen & Nemunaitis, 2006). The HSV-1 mutant HSV1716 (Table 1), deleted for both copies of ICP34.5, has shown promise in animal models of glioma (Andreansky et al., 1996), mesothelioma (Kucharczuk et al., 1997), melanoma (Randazzo et al., 1995; Randazzo et al., 1997), and lung carcinoma (Lambright et al., 1999). HSV1716 treatment caused significant tumor regression and prolonged survival. Clinical trials have been initiated for many ICP34.5-null HSV-1 vectors including HSV1716, the details of which will be discussed in section 3.

Virus	Gene(s) Mutated	Transgene	Description	Reference
First Generation				
dlsptk	TK		TK gene deletion	Martuza et al., 1991
hrR3	ICP6	LacZ	LacZ gene insertion within ICP6 coding region	Mineta et al., 1994
HSV1716	ICP34.5 (2)		deletion of both copies of ICP34.5	Randazzo et al., 1995
Second Generation				
NV1020	U_L24, ICP34.5, U_L56	TK	deletion of U_L24, U_L56, one copy of ICP34.5 exogenous TK under control of ICP4 promoter	Advani et al., 1999
G207	ICP34.5 (2), ICP6	LacZ	deletion of both copies of ICP34.5 LacZ gene insertion within ICP6 coding region	Mineta et al., 1995
G47Δ	ICP47, ICP34.5 (2)	LacZ	deletion of ICP47 and both copies of ICP34.5 LacZ gene insertion within ICP6 coding region	Todo et al., 2001b
Transcriptional Targeting				
G92A	TK, U_S3, U_L24	LacZ Alb-ICP4	based on HSV-1 d120 backbone LacZ gene insertion within TK coding regioninsertion of albumin promoter-ICP4 transgene within TK coding region	Miyatake et al., 1999
d12.CALP	TK, U_S3, U_L24	LacZ Cal-ICP4	based on HSV-1 d120 backbone E. coil LacZ gene insertion within TK coding region insertion of the CALP promoter-ICP4 transgene within TK coding region	Yamamura et al., 2001
DF3γ34.5	ICP34.5 (2)	DF3/Muc1 -ICP34.5	deletion of both copies of ICP34.5 insertion of exogenous ICP34.5 gene under control of DF3-MUC1 promoter	Chung et al., 1999
Transgene-expressing/Immunostimulatory				
rRp450	ICP6	CYP2B1	cytochrome p450 oxidase gene insertion within ICP6 coding region	Pawlik et al., 2001
HSV1γCD	ICP6	CD AFP	CD gene insertion within ICP6 coding region AFP gene insertion within ICP6 coding region	Nakamura et al., 2001
dvB7Ig	ICP34.5 (2), ICP6	LacZ B7-1	deletion in both copies of ICP34.5 LacZ gene insertion within ICP6 coding region B7-1Ig inserted within the ICP6 coding region	Ino et al., 2006
NV1034	ICP47, U_L56	LacZ GM-CSF	deletion of ICP47 and U_L56 LacZ gene insertion within ICP47 coding region GM-CSF under control of ICP6-TK hybrid promoter	Wong et al., 2001
NV1042	ICP47, U_L56, ICP34.5	LacZ TK IL-12	deletion of ICP47, U_L56, and one copy of ICP34.5 LacZ gene insertion within ICP47 coding region Rescue of TK expression through insertion of a 5.2kb sequence of HSV-2 origin at the L-S junction mIL-12 under control of the ICP6 promoter	Wong et al., 2001
OncoVEX GM-CSF	ICP47, ICP34.5 (2)	GM-CSF	deletion of ICP47 deletion of both copied of ICP34.5 GM-CSF expressed under control of the ICP47 promoter	Hu et al., 2006

Table 2. Oncolytic HSV-1 Vectors and their corresponding mutations (ICP: infected cell protein, CALP: calponin, MUC1: mucin-1, CD: cytosine deaminase, AFP: alphafetoprotein, GM-CSF: granulocyte macrophage colony-stimulating factor, IL: interleukin, TK: thymidine kinase)

Concerns over safety of using single gene deletion mutants are centered on observations of residual replication and toxicity in normal cells. The development of HSV-1 oncolytic viruses containing mutations in multiple genes increases their safety profile while also decreasing the risk of reversion to wild type. These viruses are referred to as second generation oncolytic vectors.

2.2.2 Second generation vectors

The second generation mutant G207 (Table 1) is at the forefront of oncolytic virotherapy. G207 contains deletions in both copies of ICP34.5 and a lacZ gene insertion within the ICP6 coding region. This virus selectively replicates in human tumor cells over normal cells while retaining sensitivity to antiherpetic drugs. Also, the lacZ gene insertion encodes an easily detectible histochemical maker for virus replication and spread. The safety of G207 has been demonstrated in animal models of liver (Kooby et al., 1999), ovarian (Coukos et al., 2000), melanoma (Todo et al., 2002), breast (Toda et al., 1998), colon, gallbladder, gastric (Todo et al., 2002), head and neck (Wong et al., 2001), pancreatic, prostate (Todo et al., 2002) and cervical carcinoma (Blank et al., 2002). G207 is currently in clinical trials for treatment of malignant glioma (Markert et al., 2000). Another second generation mutant, NV1020 (Table 1), is deleted for tk and one copy of ICP34.5. TK function is provided by insertion of a 5.2 kb fragment of HSV-2 DNA under the control of the ICP4 promoter (Shen & Nemunaitis, 2006). Therefore, NV1020 retains sensitivity to ACV and GCV. The efficacy of NV1020 has been demonstrated in animal models of pancreatic (McAuliffe et al., 2000), head and neck (Wong et al., 2001), and hormone-resistant prostate carcinoma (Advani et al., 1999) with the virus replicating to higher levels in comparison to mutants deleted for both copies of ICP34.5 (Bennett et al., 2002; McAuliffe et al., 2000; Advani et al., 1999). Clinical trials are currently underway using NV1020 to treat colon carcinoma liver metastases via intrahepatic arterial delivery (Kemeny et al., 2006). Furthermore, immunostimulatory HSV-1 vectors and mutants deleted for viral proteins involved in counteracting the immune response have been developed to augment antiviral and antitumor immune responses aiding in tumor regression. G47Δ (Table 1) is a derivative of G207 with an additional deletion in ICP47. Since ICP47 decreases expression of MHC I molecules through inhibition of TAP, its absence promotes tumor antigen presentation and increased antitumor immune responses (Todo et al., 2001b). In comparison to G207, G47Δ conferred enhanced inhibition of tumor growth in both immunodeficient and immunocompetent animal models while retaining the same safety profile (Todo et al., 2001b).

2.3 Transcriptional targeting

Another method of restricting virus replication to tumor cells involves insertion of tissue and/or tumor-specific promoters to drive the expression of genes which are essential to virus replication in tumor cells. This is referred to as transcriptional targeting and has shown promise as an efficient restriction mechanism. The HSV-1 mutant G92A (Table 1) places expression of the IE gene ICP4 under the control of the albumin-enhancer promoter to direct tropism to the liver and hepatocellular carcinomas (Miyatake et al., 1999). Another HSV-1 vector, d12.CALP (Table 1) expresses ICP4 under the control of the calponin promoter and selectively replicates in smooth muscle and bone tumors (Yamamura et al., 2001). In addition, a similar virus DF3γ34.5 (Table 1) expresses ICP34.5 under the control of the DF3/MUC1 enhancer/promoter and is limited to tumors expressing the DF3/MUC1

antigen, such as breast carcinomas (Kasuya et al., 2004). In an effort to direct the tropism of oncolytic herpesvirus KeM34.5 to gliomas, Kanai et al. (2007) used the musashi1 promoter to control expression of ICP34.5. Musashi1 is a neural RNA-binding protein which is used as a molecular marker for malignant glioma. In comparison to its parental vector G207, KeM34.5 showed enhanced cytotoxic ability *in vitro* as well as increased tumor regression in a murine glioma model. Recently, an oncolytic HSV-1 vector (oHSV-MDK-34.5) was developed to treat malignant peripheral nerve sheath tumors (MPNST) by fusing the human midkine (MDK) promoter to the ICP34.5 gene (Maldonado et al., 2010). Selective replication of oHSV-MDK-34.5 in MDK-positive MPNSTs resulted in antitumor effects and prolonged survival of MPNST-bearing nude mice. Oncolytic adenoviruses have been engineered with restricted expression of E1A and E1B genes by placing them under the control of the prostate-specific antigen (PSA) and probasin promoters in CV706 and CV787 respectively (Chen et al., 2001; Yu et al., 1999). For instance, the replication of CV706 was found to be highest in PSA-producing tissues and was able to induce cytotoxic effects in infected tumor cells (Rodriguez et al., 1997).

The differential expression of micro-RNAs (miR) between normal and tumor cell types has been exploited to generate oncolytic HSV-1 vectors which selectively replicate in the absence of select miRs. The expression of miR-143 and miR-145 is significantly down-regulated in prostate carcinomas in comparison to normal healthy tissue. Target sequences for miR-143 or miR-145 were incorporated into the 3'-untranslated region of ICP4 to generate the oncolytic HSV-1 vectors CMV-ICP4-143T and CMV-ICP4-145T (Lee et al., 2009). Tumor-specific replication resulted in significant reductions in tumor volume and enhanced survival in mice bearing LNCaP human prostate tumors.

2.4 Cellular targeting

Modification of viral entry proteins, referred to as cellular targeting, is another method by which the replication of oncolytic vectors can be restricted to certain tissues and tumor cells. The recombinant HSV-1 virus, R5141, was engineered to enter cells solely via the IL-13Rα2 receptor (Zhou et al., 2006). Briefly, the natural binding sites for HSPGs in gB and gC were replaced with the amino acid sequences encoding IL-13. Multiple amino acid substitutions were made in and around the HveA and nectin-1 binding sites to abolish their interaction with gD. An additional vector developed by the same group, R5181, enters cells via the urokinase plasminogen activator receptor. These studies demonstrate the application of this method to multiple receptor classes (Kamiyama et al., 2006). Although the mechanism of HSV-1 entry has not been fully elucidated, the upregulation of receptors on cancer cells, such as epidermal growth factor receptor (EGFR), folate receptor, CD44, CD38 and others, provides the basis for this approach but requires further study to achieve full tumor selectivity.

2.5 Armed oncolytic vectors

Although oncolytic vectors show promise as novel cancer therapeutics, studies indicate that in the majority of cases virus replication on its own is insufficient to induce full tumor regression. Thus, enhanced cytotoxic effects are desired and have been achieved through the expression of 'suicide' and immunostimulatory genes.

Introduction of genes encoding immunostimulatory molecules and prodrug-converting enzymes have been used to enhance the cytolytic ability of oncolytic viruses and augment the antitumor immune response leading to increased tumor regression and survival. Initial

'suicide' gene therapy vectors focused on expressing HSV-1 tk to infer sensitivity to GCV. However, this method inhibited virus replication at early stages and therefore was not efficient. As a result, expression of therapeutic genes are generally placed under the control of a late viral promoter to allow for sufficient virus replication to occur before induction of cytolytic effects that would otherwise halt replication prematurely.

Studies in immunocompetent animals have highlighted a bimodal mechanism of tumor clearance: tumor cell lysis as a result of oncolytic virus replication and induction of an antitumor immune response. Direct injection or use of oncolytic vectors expressing immunostimulatory cytokines has been used to induce inflammatory and tumor antigen-specific responses. Several studies describe vectors expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) as an immunostimulant. GM-CSF induces myeloid precursor cell activation, recruits DCs, and has been used in tumor cell vaccines resulting in increased therapeutic benefits (Toda et al., 2000; Nemunaitis, 2005; Eager et al., 2005). For example, treatment with OncoVEX$^{GM\text{-}CSF}$ (Table 1), a second generation HSV-1 vector, resulted in tumoristatic effects on injected and distal tumors in patients with cutaneous or subcutaneous breast, head and neck, gastrointestinal and malignant melanoma (Hu et al., 2006). HSV-1 constructs NV1034 and NV1042 (Table 1) , expressing GM-CSF or IL-12 respectively, prolonged survival and caused tumor regression in murine models of squamous cell carcinoma (Wong et al., 2001). NV1042 treatment resulted in an enhanced antitumor effect in comparison to NV1034 and a control vector NV1023, which does not express cytokines (Wong et al., 2001). Oncolytic virus treatment was accompanied by the induction of a strong inflammatory response, including accumulation of CD4+ and CD8+ T cells (Wong et al., 2001) and inhibition of tumor angiogenesis (Wong et al., 2004). Overall, 57% of mice treated with NV1042 were refractory to tumor re-challenge, in comparison to 14% of NV1034 and NV1023-treated animals (Wong et al., 2001). These results suggest the establishment of an antitumor memory immune response. The B7 family of membrane proteins, B7-1 (CD80) and B7–2 (CD86), are potent co-stimulatory molecules and interact with CD28 and CTLA-4 (CD152) on the T-cell surface. A HSV-1 vector expressing the B7-1-immunoglobulin (B7-1-Ig) fusion transgene (dvB7Ig) (Table 1) has been studied in a murine model of neuroblastoma (Todo et al., 2001a). Intraneoplastic inoculation of dvB7Ig inhibited tumor growth and prolonged the survival of mice bearing intracerebral tumors. A significant CD4+ and CD8+ T-cell response was observed, with immunodepletion studies indicating that CD8+ T cells play a key role in the antitumor response. Cured animals were refractory to tumor re-challenge indicating establishment of an antitumor memory immune response.

Prodrug-converting enzymes such as cytosine deaminase (CD) and cytochrome p450 oxidase have been used to induce the expression of noxious compounds to enhance oncolytic cytotoxicity. Yeast CD converts the prodrug 5-fluorocytosine to 5-fluorouracil, which inhibits DNA synthesis. HSV1γCD (Table 1), in which ICP6 is inactivated by insertion of CD, has shown promise in murine models of liver metastases (Nakamura et al., 2001). Expression of CD prolonged survival and enhanced tumor regression without significantly affecting virus replication and oncolysis. Furthermore, similar results were obtained using rRp450 (Table 1) a HSV-1 mutant expressing the CYP2B1 transgene which codes for cytochrome p450 oxidase and converts cyclophosphoramide (CPA) into phosphoramide mustard. Despite the immunosuppressive activity of CPA, its expression enhanced the cytolytic effect of rRp450 against hepatocellular carcinoma both *in vitro* and *in vivo* without prematurely inhibiting HSV-1 replication (Pawlik et al., 2002).

3. Clinical trials

The antitumor efficacy of oncolytic herpesviruses has been demonstrated in preclinical animal models. However, evaluation of tumor-selectivity, virus replication and spread must be evaluated in humans. Clinical trials have been completed, or are in progress, for NV1020, HSV1716, G207, OncoVEX$^{GM\text{-}CSF}$ and HF10 (Table 2).

3.1 NV1020 clinical trials

The liver is the most common site of colorectal metastases. It has been estimated that 60% of patients with metastatic colorectal cancer have liver-predominant disease which is the limiting factor determining patient survival (Kemeny et al., 1994; Rosen et al., 1995). Currently, chemotherapy is the first line of treatment prescribed to patients, but rarely produces significant antitumor results (Meyers et al., 2003). Second line therapies are thus pivotal in the treatment of colorectal liver metastases.

In a phase I dose-escalating study twelve patients were divided equally into four cohorts receiving $3x10^6$ plaque forming units (pfu), $1x10^7$ pfu, $3x10^7$ pfu or $1x10^8$ pfu NV1020 via hepatic arterial infusion (Kemeny et al., 2006). NV1020 treatment was well tolerated; however, adverse events associated with virus administration occurred and included pyrexia, headache and rigors. Within the first forty-eight hours, minor elevations in liver enzymes occurred but were self-limiting without intervention and normalized shortly thereafter. Hepatic artery samples indicated viral clearance in all participants, including those receiving a dose of $1x10^8$ pfu. With the exception of one patient, PCR analysis did not detect HSV DNA in saliva, urine, conjunctiva, vaginal, and serum samples. The HSV-positive patient did not experience any HSV-specific adverse effects during the course of the study. Furthermore, levels of serum cytokines, including IL-1, IL-2, IFNγ, and TNFα, as well as cytotoxic T cell counts were measured one week following NV1020 treatment. Minor increases in serum IL-1, IL-2, IFNγ and TNFα occurred but with no apparent relationship to virus administration. Variations in cytotoxic T cell counts were minor and inconsistent among patients. Overall, at twenty-eight days post-treatment two patients exhibited 39% and 20% reductions in tumor size. Of the remaining patients, three showed tumor progression while seven achieved tumor stabilization. This study established that hepatic arterial infusion of NV1020 holds potential as a treatment for colorectal liver metastases.

The phase I study by Kemeny et al. (2006) was used as the foundation for a multicentre phase I/II study evaluating the safety and antitumor effects of multiple doses of NV1020 in patients with colorectal liver metastases (Geevarghese et al., 2010). A total of thirteen and nineteen patients were enrolled in the phase I and II trials respectively. Each patient received four doses of NV1020 via hepatic arterial infusion followed by chemotherapy. The phase I cohort received doses of $3x10^6$ pfu, $1x10^7$ pfu, $3x10^7$ pfu, and $1x10^8$ pfu to determine the optimal dose for use in phase II trials. Adverse events associated with virus treatment were mild and did not involve altered liver function. PCR analysis failed to detect NV1020 DNA in the serum, genital swabs, and saliva of patients. However, HSV-1 DNA was present in the samples of two patients but no HSV-related symptoms were observed. Moreover, serological levels of IL-6, TNFα and IFNγ transiently increased in a dose-dependent manor. Following NV1020 administration, 50% of patients exhibited stable disease and after subsequent chemotherapy 68% maintained this status. The one year survival was 47.2%, a significant increase compared to conventional outcomes. A phase III trial is currently underway for NV1020 treatment of colorectal liver metastases.

Virus	Cancer Type	Phase	Participants	Delivery	Reference
NV1020	Colorectal liver metastases	I	12	hepatic artery	Kemeny et al., 2006
	Colorectal liver metastases	I	13	hepatic artery	Geeverghese et al., 2010
	Colorectal liver metastases	II	19	hepatic artery	Geeverghese et al., 2010
HSV1716	Recurrent glioma	I	9	intratumoral	Rampling et al., 2000
	High-grade glioma	I	12	intratumoral	Papanastassiou et al., 2002
	Recurrent glioma	I	12	intratumoral	Harrow et al., 2004
	Malignant Melanoma	I	5	intratumoral	MacKie et al., 2001
	HNSCC	I	20	intratumoral	Mace et al., 2008
G207	Malignant glioma	I	21	intratumoral	Markert et al., 2000
	Malignant glioma	Ib	6	intratumoral	Markert et al., 2009
	Recurrent glioma	I	9	intratumoral	Karrasch et al., 2009
$OncoVEX^{GM-CSF}$	Solid tumors	I	30	intratumoral	Hu et al., 2006
	HNSCC	I/II	17	intratumoral	Harrington et al., 2010b
	Metastatic melanoma	II	50	intratumoral	Senzer et al., 2009
HF10	Reccurent breast carcinoma, pancreatic carcinoma	I	9	intratumoral	Nakao et al., 2007
	pancreatic carcinoma	I	6	intratumoral	Nakao et al., 2011

Table 2. Clinical Trials with Oncolytic Herpes Simplex Viruses (HNSCC; head and neck squamous cell carcinoma)

3.2 HSV1716 clinical trials

The use of HSV1716 in the treatment of patients with recurrent glioma and high grade glioma (HGG) has been evaluated in clinical trials. Current therapies for malignant glioma, including radiotherapy and chemotherapy, provide a median survival time of

only 1 year following diagnosis (Rampling et al., 1997; Rampling et al., 1998; Subach et al., 1999). Patient survival following primary therapy is a staggering 5 months (Rajan et al., 1994), warranting development of novel treatments which will prolong survival and improve patient outcomes. Preclinical data indicates the ability of HSV1716 to replicate in a wide variety of tumor types including those of central nervous system (CNS) origin (Brown et al., 1994; Randazzo et al., 1995). In murine models of malignant glioma HSV1716 induced significant tumor regression with minimal effect on normal CNS tissue (McKie et al., 1998).

In a phase I dose escalation study nine patients with recurrent malignant glioma received an intratumoral injection of HSV1716 at doses ranging from $1x10^3$-$1x10^5$ pfu (Rampling et al., 2000). All patients had previously undergone radiotherapy and/or surgery and had since relapsed. Virus treatments were well tolerated with no adverse effects. In addition, PCR analysis failed to detect shed HSV1716 or HSV-1 DNA in patient buccal and serum samples. Furthermore, HSV-1 reactivation in the form of skin lesions did not occur. After 14 months, four patients remained alive with two in complete remission. This study demonstrated the feasibility and safety of HSV1716 for treatment of recurrent malignant glioma and constituted the framework for subsequent phase II clinical trials.

An additional proof of principle study evaluated the toxicity and replication of HSV1716 in patients with HGG (Papanastassiou et al., 2002). Twelve patients with HGG received an intratumoral injection of HSV1716 at $1x10^5$ pfu. No adverse effects were observed following injection of the virus or throughout the duration of the study. At four to nine days post-injection, tumors were removed and assayed for the presence of virus. The tumors removed from two participants contained a higher concentration of virus than was injected and contained HSV-specific antigens. It should be noted that these patients were seronegative at the time of injection and subsequently seroconverted. Furthermore, virus replication was not detected in tumors of seropositive patients which could be the result of virus neutralization during the tumor resection process. However, PCR analysis detected HSV1716 DNA at the injection site of ten patients and at distal sites in four, including seropositive individuals. Together these data demonstrate that HSV1716 was able to selectively replicate in patient gliomas and spread to distal tumor sites despite the presence of pre-existing immunity in seropositive individuals. Further to the first study, this clinical trial added to the safety profile of HSV1716 for use in the treatment of malignant glioma.

In the third clinical trial with HSV1716 for treatment of HGG, the virus was used to clear residual tumor cells after resection (Harrow et al., 2004). Following tumor resection, residual tumor cells remain within the resection cavity and ultimately divide to form a new tumor mass. A total of twelve patients were enrolled in the trial, six with recurrent disease and six newly diagnosed. Following tumor resection patients were injected with $1x10^5$ pfu HSV17161 into adjacent brain tissue. In addition, eight to ten sites adjacent to the tumor bed were also injected. All patients received subsequent radiotherapy or chemotherapy. Magnetic resonance imaging (MRI) or computed tomography (CT) scans were taken pre- and postoperatively to monitor tumor regression and the structural anatomy of the surrounding brain tissue. Patients also underwent single photon emission computed tomography (SPECT) to identify HGG growth as indicated by areas of high cellular metabolic activity. At the time of tumor resection, patient tumor cells were collected for *ex vivo* HSV1716 replication analysis. Results indicate the ability of HSV1716 to replicate in HGG tumor cells. No significant adverse effects or toxicity associated with HSV1716

treatment was observed. Overall, three patients were clinically stable up to twenty-two months post-treatment. Tumor regression was evident in the patient alive after twenty-two months as indicated by imaging analysis. The lack of viral-mediated toxicity post-HSV1716 injection into normal brain tissue demonstrates the utility of this virus for use in combination therapy for patients with HGG.

Preclinical studies indicate the ability of HSV1716 to selectively replicate in human melanoma cells *in vitro* and cause tumor regression in murine models of melanoma (MacKie et al., 2001). In a pilot study to ascertain the efficacy of HSV1716 treatment for melanoma, five HSV-1 seropositive patients with stage four melanoma received intratumoral injections of HSV1716 at $1x10^3$ pfu (MacKie et al., 2001). Two patients received one injection, two received two injections and one received four injections. No adverse effects were observed and anti-HSV antibody titres did not significantly increase with treatment. Injected nodules were excised at fourteen (single injection) or twenty one (multiple injections) days post-injection for assessment of pathology and virus replication. One patient showed flattening of tumor nodules twenty-one days post-injection while all participants had evidence of tumor necrosis. There was no indication of pathogenesis or necrosis in adjacent normal melanocytes. This study established the utility of HSV1716 as a treatment for melanoma, with replication and associated pathology restricted to tumor cells.

Comparable to malignant glioma, the five year survival rates for patients with head and neck squamous cell carcinoma (HNSCC) ranges from 0-40% depending on localization of the tumor (Forastiere et al., 1998). Patient relapse rates and the high incidence of distant metastases makes HNSCC a formidable challenge requiring novel therapeutic options. A total of twenty patients with oral HNSCC received intratumoral injections of $1x10^5$ pfu (five patients) or $5x10^5$ pfu (fifteen patients) HSV1716 (Mace et al., 2008). Injections were well tolerated with no adverse effects associated with virus treatment. Of the patients enrolled in the study two were seronegative, but both seroconverted within a week of HSV1716 injection. However, the seropositive cohort did not experience changes in anti-HSV antibody levels post-injection. Interestingly, PCR analysis detected HSV DNA in the blood of all patients receiving the lower dose of $1x10^5$ pfu; however, no infectious virus was recovered from any tissue sample as determined by an infectious plaque assay. Tumor histology was negative for inflammation and necrosis at injection sites but infiltration of inflammatory lymphocytes was apparent. Although significant tumor regression was not observed in this trial, results warrant a dose escalation study due to the established safety profile of HSV1716 for treatment of HNSCC.

3.3 G207 Cclinical trials

The HSV-1 mutant G207 has shown exceptional promise in clinical trials for the treatment of malignant glioma. Preclinical data shows that G207 is able to selectively replicate in tumor cells from a variety of histological origins including gliomas (Mineta et al., 1995). Furthermore, the non-pathogenic nature of G207 was confirmed through intracerebral injection of the virus into HSV-susceptible mice and simian primates (Hunter et al., 1999; Mineta et al., 1995). These data led to evaluation of G207 in a phase I dose escalation trial for the treatment of malignant glioma (Markert et al., 2000). Twenty one patients were enrolled in the study and received one of three intratumoral dosing regimens. Patients in the first cohort received doses ranging from $1x10^6$ to $1x10^8$ pfu, the second cohort received a single dose of $1x10^9$ pfu, and the third cohort received a dose of $3x10^9$ pfu at five distinct sites within the tumor mass. Three patients were included in each dose cohort and injection sites

were determined by contrast enhanced areas indicated on CT scans. All doses of G207 were well tolerated with no serious adverse effects associated with virus inoculation. Tumor biopsies were performed post-injection on six patients, and brains were obtained upon autopsy from five deceased patients. PCR analysis indicated the presence of G207 DNA in resected tumors obtained from patients at 56 and 157 days post-injection. However, viral shedding was absent from all patient saliva samples. Tumor regression was seen in eight patients ranging from four days to one month post-injection. Two patients have survived over four years and one patient died from causes unrelated to G207 treatment. The promising results borne from this trial are the cornerstones for design of a phase 1b trial involving G207 treatment of recurrent malignant glioma followed by tumor resection and tumor bed inoculation.

Objectives of the phase 1b clinical trial were four fold (Markert et al., 2009). The safety of two intratumoral injections within a one week period was ascertained as well as the safety associated with direct injection of G207 into normal brain tissue. Furthermore, virus replication and the presence of anti-HSV immune effectors were measured in inoculated tumors. Six patients were enrolled in the study, each receiving two doses of G207 totalling $1.5x10^9$ pfu. Specifically, an initial intratumoral injection of $1.5x10^8$ pfu was delivered via catheter followed by tumor resection two to five days later. Subsequent injections of G207 were administered into brain tissue surrounding the resection cavity. Treatment was well tolerated with most adverse effects unrelated to G207 inoculation. PCR analysis did not detect HSV DNA in patient saliva, urine, conjunctiva and serum samples despite the presence of virus replication in resected tumor samples from three patients. Inflammation was not present at the inoculation site as determined through patient MRIs, and no patients developed viral encephalitis, even after two injections of G207. Tumor samples were obtained from one patient pre- and post-G207 treatment for immunohistochemical analysis. Prior to treatment only a small population of immune effectors, including lymphocytes and macrophages, were present in the tumor. However, two days post G207 injection there was significant infiltration of mature T cells including cytotoxic T cells, monocytes, macrophages, microglia, and NK cells (Figure 3). The presence of B cells was also detected in the tumor sample but at much lower levels. All seronegative patients subsequently seroconverted following G207 treatment and half of seropositive participants experienced an increase in anti-HSV antibody titres. Due to the small sample size no conclusions regarding the efficacy of G207 treatment can be drawn. However, according to MRI analysis no participants achieved a complete or partial response, defined as a 50% decrease in contrast enhancing tumor volume following G207 administration. Results from this study warrant the progression of G207 therapy for malignant glioma to phase II clinical trials.

A combination therapy study for the treatment of recurrent malignant glioma demonstrated an additive effect of G207 and radiotherapy (Karrasch et al., 2009). In a phase I clinical trial, nine patients received intratumoral injections of $1x10^9$ pfu G207 followed by focal radiotherapy (5 Gray) twenty four hours later. Combination therapy was well tolerated and dose-limiting toxicities were not reached. Patients achieving a partial response were re-treated with G207 and radiotherapy one month following initial treatment due to tumor recurrence. The median survival times for patients suffering from relapsed gliomablastoma multiforme and anaplastic astrocytoma was 7.4 and 9.25 months, respectively. HSV DNA was detected within tumors indicating intratumoral G207 replication. Overall, G207 combination therapy prolonged patient survival over traditional treatment modalities.

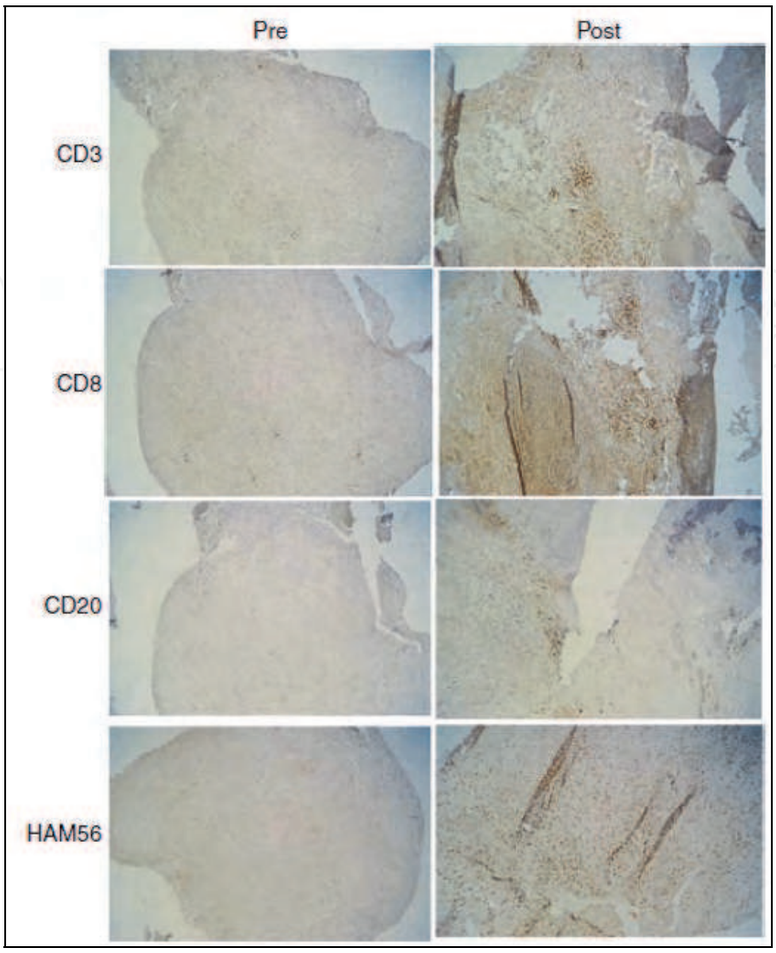

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Fig. 3. Tumor-infiltrating lymphocytes and macrophages in patient tumor tissue pre- and post-G207 treatment. Immunohistochemical staining was performed on paraffin blocks of patient tumor samples pre- and post-G207 treatment using antibodies against CD3 (infiltrating T cells), CD8 (cytotoxic and suppressor T cells), CD20 (B cells), and HAM56 (monocyte/macrophage).

3.4 OncoVEX$^{GM\text{-}CSF}$ clinical trials

Generation of OncoVEX$^{GM\text{-}CSF}$ was prompted by the success of other HSV vectors as well as the need for increased antitumor immune activity during therapy.

A phase I study involving thirty patients with breast, head and neck, gastrointestinal, and melanoma malignancies evaluated the safety and antitumor activity of OncoVEX$^{GM\text{-}CSF}$ (Hu et al., 2006). Participants were divided into two cohorts, thirteen received a single intratumoral injection at one of three dose levels ($1x10^6$ pfu, $1x10^7$ pfu and $1x10^8$ pfu), and the remaining seventeen were placed in a multi-dose group and received escalating doses of virus every one to three weeks. All doses were well tolerated with no adverse events associated with virus treatment occurring in either the single or multi-dose groups. However, seronegative participants experienced more pronounced injection site irritation

which eventually cleared without intervention. It was also observed that all seronegative individuals seroconverted within three to four weeks of their first injection of OncoVEX$^{GM\text{-}CSF}$. Transient elevations in anti-HSV antibody titres occurred in seropositive patients but did not limit therapeutic efficacy. PCR analysis detected HSV DNA in the blood of two patients in the single dose cohort (eight hours and one week post-injection) and in eight patients in the multi-dose cohort (one and eight hours post-injection). Low levels of HSV DNA were detected in the urine of two patients in the single dose cohort (eight hours and one week post-injection), and no patients tested positive in the multi-dose cohort. Viral DNA was not detected at any other time points during the study. Tumor biopsies indicated the infiltration of immune effectors following OncoVEX$^{GM\text{-}CSF}$ injection, including increases in T cell and macrophage populations. Areas of tumor necrosis were found to correspond to sites of virus injection, while normal tissue showed no evidence of necrosis. Stable disease was detected in three patients, two with melanoma and one with breast cancer. Furthermore, the injected tumors of several patients with metastatic melanoma regressed or were cleared but other metastatic lesions appeared at distal sites. No partial or complete responses were reached in this study but the safety and tumoristatic effects of OncoVEX$^{GM\text{-}CSF}$ treatment on injected and distal tumors was established.

The phase I trial by Hu et al. (2006) established the safety and efficacy of OncoVEX$^{GM\text{-}CSF}$ for the treatment patients with metastatic melanoma, laying the ground work for a phase II clinical trial (Senzer et al., 2009). A total of fifty patients with stage IIIc (10) or stage IV (40) metastatic melanoma received an initial intratumoral injection of OncoVEX$^{GM\text{-}CSF}$ at $1x10^6$ pfu to seroconvert patients who were seronegative and to reduce adverse effects associated with virus injection, as primarily seen in seronegative patients. Three weeks later patients received another intratumoral injection at $1x10^8$ pfu, which was repeated every two weeks thereafter. Tumors were injected based on size with a maximum of ten treated per visit. The adverse effects associated with OncoVEX$^{GM\text{-}CSF}$ administration were primarily mild in nature. However, ten patients experienced fatigue, dyspnea, and pain which was possibly disease related. Injection site swabs and urine were collected from patients and analyzed for the presence of HSV DNA by PCR. Analysis detected HSV DNA from the swab of one patient at very low levels, while no urine contained viral DNA. Local effects on OncoVEX$^{GM\text{-}CSF}$-injected tumors were apparent after as few as two treatments, but regression of distal tumors typically occurred twelve months after the first treatment. Overall, ten patients achieved complete response without additional surgery and thirteen did not have evidence of disease after further surgical tumor resection. Interestingly, HSV serostatus did not seem to play a role in response to OncoVEX$^{GM\text{-}CSF}$ therapy. The prolonged survival (>1 year) of patients enrolled in this study and the antitumor activity of OncoVEX$^{GM\text{-}CSF}$ prompted approval of a phase III study, which is currently underway.

A study by Kaufman et al. (2010) evaluated local and distal immune responses in patients enrolled in the phase II Senzer et al. (2009) clinical trial. Peripheral blood and tumor samples were analyzed for the presence of myeloid-derived suppressive cells (MDSC), regulatory T cells (Treg, CD4+ FOXP3+) and suppressor T cells (Ts, CD8+ FOXP3+). Significant increases in the level of peripheral and tumor-associated antigen-specific T cells (MART-1) were observed following OncoVEX$^{GM\text{-}CSF}$ injection. Notable decreases in Treg and Ts cell populations occurred in injected tumors in comparison to non-injected distal tumors. This may be due to decreases in MDSCs which have been shown to negatively regulate activator T cell activity by diverting them to a regulatory phenotype.

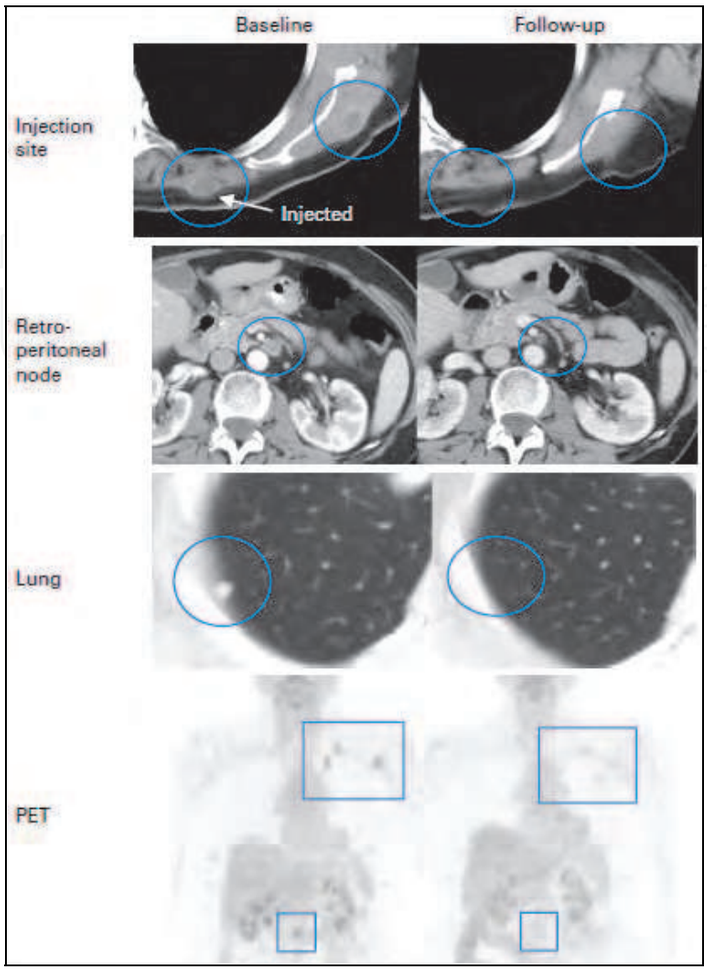

Fig. 4. Patient 703 Response to OncoVEX$^{GM\text{-}CSF}$ Therapy. CT images of injections sites in the left shoulder, retroperitoneal lymph node, and lungs at baseline (left) and three months (right). Positron emission tomography (PET) image at baseline (left) and at eight months (right). Highlighted areas indicate lesions before and after OncoVEX$^{GM\text{-}CSF}$ injection.

A combination therapy phase I/II trial for the treatment of stage III/IVA head and neck squamous cell carcinoma demonstrated an additive effect of OncoVEX$^{GM\text{-}CSF}$ and chemo radiotherapy (CRT) (Harrington et al., 2010b). Seventeen patients were enrolled in the study and received dose escalating OncoVEX$^{GM\text{-}CSF}$ (cohort one: four injections at $1x10^6$ pfu; cohort two: $1x10^6$ pfu followed by three injections at $1x10^7$ pfu; cohort three: $1x10^6$ pfu followed by three injection at $1x10^8$ pfu) intratumoral injections on days one, twenty two, forty three and sixty four. Patients were treated with full-dose radical CRT comprised of radiotherapy (70 Gray) in thirty five daily fractions over seven weeks with cisplatin administered on days one, twenty one and forty two at 100 mg/m^2 body surface area. Treatment was well tolerated and dose-limiting toxicities were not identified in any cohorts. Viral shedding was detected at transient low levels in three patients. HSV DNA was detected at levels higher than the input dose in injected and adjacent uninjected tumors by immunohistochemistry

and PCR analysis. All seronegative patients seroconverted and increased antibody titres were measured in all patients in a dose-dependent manor. Overall, thirteen patients achieved partial or complete response including five whom achieved complete response following only two or three doses of OncoVEX$^{GM\text{-}CSF}$. All patients achieved loco-regional control with 70.5% remaining alive and in complete remission at the time of publication. A phase III study is currently underway.

3.5 HF10 clinical trials

The HF10 oncolytic vector is a naturally mutated clonal derivative of patient strain HF which contains inactivating mutations in U_L43, $U_L49.5$, U_L55, U_L56 and LAT coding sequences (Takakuwa et al., 2003). Although the function of U_L56 is unknown, U_L43, $U_L49.5$ and U_L55, encode membrane, envelope and tegument proteins, respectively. Preclinical data has shown that HF10 therapy causes significant tumor regression and prolongs survival in animal models of breast and colon carcinoma (Kimata et al., 2003; Teshigahara et al., 2004). Furthermore, cured mice were refractory to tumor re-challenge indicating that an antitumor immune response was established.

A phase I trial to evaluate the safety and antitumor activity of HF10 was performed on six patients with recurrent breast carcinoma (Nakao et al., 2007). Patients were chosen with metastatic skin lesions to facilitate administration of the virus subcutaneously. Patients received $1x10^4$ pfu, $1x10^5$ pfu or $1x10^5$ pfu for three days, $5x10^5$ pfu or $5x10^5$ pfu for three days. Tumor nodules were excised fourteen days post-injection for evaluation of virus pathology and replication. The same treatment regimen was adopted for three patients with non-resectable pancreatic carcinoma except HF10 was delivered via intratumoral catheter. HF10 therapy was well tolerated with no adverse events associated with virus administration in either the breast or pancreatic carcinoma participants. Analysis of excised tumor samples revealed significant decreases in tumor cell viability (30-100%) associated with virus replication. Tumor regression was evident in the skin nodule of one patient with recurrent breast carcinoma, but no other significant decreases in tumor volume were noted.

An additional phase I clinical trial was performed on six patients with non-resectable pancreatic tumors caused by liver metastases and invasion of lymph nodes surrounding the aorta (Nakao et al., 2011). Intratumoral injection of HF10 ($1x10^6$ pfu) was performed at four sites during laparotomy or with the aid of CT imaging. An intratumoral catheter was inserted during surgery for subsequent delivery of three daily doses of HF10 for three days. No adverse effects associated with HF10 treatment were observed during the trial. PCR analysis failed to detect HSV DNA in blood samples and abdominal cavities of patients, but prolonged viral expression was evident in several patient tumors up to 318 days post-injection. Moreover, analysis of resected tumor samples indicated significant infiltration of macrophages, CD4+ and CD8+ T cells into HF10-injected tumor tissue. Upon completion of this trial four patients achieved partial response while the remaining two maintained progressive disease status. Together, these studies demonstrate the efficacy of HF10 for use in oncolytic virotherapy for the treatment of patients with breast and pancreatic carcinoma.

4. Challenges with oncolytic virotherapy

The safety and antitumor efficacy of HSV-1 vectors in clinical trials has inspired the development of treatment platforms using engineered and natural oncolytic viruses, as well

as combination therapy trials. Despite promising results in preclinical studies, *in vitro* assays do not always predict *in vivo* outcomes. These discrepancies can be attributed to the challenges of adapting therapy from cell culture to *in vivo* tumors which contain a plethora of hurdles to both virus replication and tumor clearance.

4.1 The tumor microenvironment

The heterogeneity of the tumor mass, including necrotic foci, normal stroma, the basal membrane and extracellular matrix (ECM), presents a formidable road block to virus penetration. Furthermore, viral infection often elicits host inflammatory responses resulting in increased recruitment of immune effectors. Overall, the effects of the tumor microenvironment may counteract the antitumor effects of oncolytic virotherapy.

The ECM forms a complex molecular sieve which regulates cell signalling processes between tumor and normal stromal cells. Modifications to the ECM impact tumor biology and accessibility, and therefore the response to therapy. McKee et al. (2006) demonstrated that fibrillar collagen acts as a barrier to HSV-1 vector MGH2 spread within melanoma. Viral particles localized to collagen-free areas of the tumor with limited spread to collagen-rich regions. Co-treatment of tumors with MGH2 and collagenase enhanced virus spread and tumor regression. In addition, viruses expressing enzymes such as matrix metalloproteinases (MMPs), which degrade ECM components, could be used. Chondroitinase ABC (Chase-ABC) is a bacterial enzyme which cleaves chondroitin sulphate glycosaminoglycans from proteoglycans. In comparison to the control (rHsvQ), the oncolytic HSV-1 vector expressing Chase-ABC (OV-Chase) enhanced viral spread, inhibited tumor growth, and prolonged survival of animals with subcutaneous and intracranial glioma xenografts (Dmitrieva et al., 2011). Furthermore, the ectopic expression of MMP-9 on murine malignant glioma xenografts has been shown to increase spread of the oncolytic HSV-1 vector JD0G through degradation of collagen type IV, a major component of the ECM and basal membrane.

Angiogenesis is crucial to tumor growth as vasculature provides a network from which cells obtain oxygen, metabolites and dispose of waste. Disruption of the balance between pro- and anti-angiogenic factors is caused by changes in the tumor microenvironment such as hypoxia, acidosis, inflammation and gene mutations. Ultimately, an 'angiogenic switch' occurs which leads to increased synthesis of vasculature supporting rapid tumor growth. Necrotic, acidotic, and hypoxic areas result, in part, from abnormalities in tumor vasculature such as aberrations in the morphology of endothelial cells and mechanical stress on developing vessels from rapidly proliferating tumor cells. Infection of tumor cells with oncolytic viruses can have both pro- and anti-angiogenic effects. Infection of ovarian tumor endothelial cells with HSV1716 has been shown to disrupt tumor vasculature (Benencia et al., 2005). Conversely, infection of human glioma cells with G207 reduces expression of thrombospondin, resulting in increased vasculature (Aghi et al., 2007). This effect can be countered through the administration of thrombospondin-derived peptides. In models of glioblastoma, cases where oncolytic virus infection results in an anti-angiogenic response, adjacent virus-free areas have been shown to upregulate angiogenesis (Huszthy et al., 2010). Furthermore, increases in tumor angiogenesis can facilitate virus-induced inflammatory responses. The resultant changes in vascular perfusion can lead to hypoxia-mediated killing of uninfected tumor cells (Breitbach et al., 2007) and increased vascular leakage due to vascular shutdown and hyperpermeability, respectively (Kurozumi et al., 2007). The impact of vascularity on oncolytic virotherapy and vice versa has inspired combination therapy

trials using anti-angiogenic drugs such as Bevacizumab (Willett et al., 2004), Trichostatin A (Liu et al., 2008) and Cilengitide (Kurozumi et al., 2007). In addition, oncolytic vectors expressing anti-angiogenic genes have been engineered to modulate the activity of factors such as vascular epithelial growth factor (VEGF), MMPs, fibroblast growth factors (FGF), and interleukins. Rapid anti-angiogenesis mediated by oncolytic virus (RAMBO), a novel HSV-1 vector expressing vasculostatin, a brain-specific angiostatic peptide, has been used in the treatment of subcutaneous and intracranial gliomas. Compared to a control virus, intratumoral injection of RAMBO resulted in tumor regression and enhanced antitumor effects including decreased tumor vascularity, microvessel density, and angiogenesis (Hardcastle et al., 2010).

4.2 Virus delivery

The importance of diffuse inoculation has been illustrated in mathematical models of virus replication. Three methods of intratumoral inoculation including injection into the entire tumor, the tumor core and the tumor rim were analyzed for their ability to eradicate the entire tumor in the presence of necrosis and an innate immune response (Wein et al., 2003). Data from preclinical and clinical trials were used to design the model and identify parameter values. Even in the absence of an innate immune response, complete tumor regression requires widespread distribution of the virus achieved by multiple injections into the entire tumor. However, an antiviral innate immune response can prevent tumor eradication, warranting methods for immune evasion to ensure efficient virus spread. While intratumoral injection is feasible for easily accessible and solid tumors, systemic delivery options are necessary for the treatment of metastatic disease and non-accessible tumors. Virus inactivation by neutralizing antibodies, innate immune responses, complement-mediated opsonisation, and insufficient adsorption limit the efficacy of systemic delivery options. Delivery of oncolytic vectors within a collagen matrix or liposomes could be used to minimize premature inactivation by immune surveillance mechanisms. Alternatively, transient immunosuppression using antibody neutralizing compounds, inhibiting B cell maturation or antibody-receptor interactions may allow for efficient vector delivery. Administration of anti-CD20 antibodies, CPA, and plasmapheresis has been used to inhibit immunoglobulin production by B cells and counteract the neutralizing activity of antiviral antibodies (Ikeda et al., 2000; Vile et al., 2002). For instance, pretreatments of cobra venom factor (CVF) and CPA followed by intravascular injection of hrR3 leads to increased viral spread and prolonged survival in a rat model of malignant glioma (Ikeda et al., 2000). Although studies have indicated that pre-existing antibodies to HSV-1 do not limit antitumor efficacy of oncolytic virus treatment, seronegative patients have a higher incidence of adverse effects from virus inoculation (Chahlavi et al., 1999; Lambright et al., 2000; Hu et al., 2006). In a phase II study for the treatment of metastatic melanoma, seronegative participants received an initial intratumoral injection of OncoVEX$^{GM\text{-}CSF}$ to induce seroconvertion (Senzer et al., 2009). This pre-treatment reduced adverse effects associated with virus injection.

Although increasing input dose is a tempting strategy to augment low infectivity from vector loss during systemic delivery, current manufacturing processes limit attainable HSV titres to $3x10^9$ pfu/mL (Varghese & Rabkin, 2002). The use of a wild type virus, which does not require attenuation to achieve selective oncolysis, could facilitate manufacturing processes and yield higher titres. The use of replication-competent vectors is another method by which the input dose can be amplified. However, care should be taken to direct the tropism of such viruses to preclude unwanted replication within normal healthy tissue.

4.3 The immune response

The infiltration of host immune cells into the tumor microenvironment is an important factor modulating the success of oncolytic virotherapy. Innate and adaptive immune responses can limit virus spread resulting in suboptimal tumor clearance and persistence of residual tumor cells. Treatment of metastatic glioma using oncolytic HSV-1 vectors results in an inflammatory response and increased vasodilation induced by expression of IFNγ and ISGs (Kurozumi et al., 2007). This prompts the infiltration of innate and adaptive immune cells causing a decrease in virus replication and spread. Controlling the entry of immune cells into the tumor microenvironment can be used to increase virus replication. Analysis of glioma tissue samples treated with hrR3 indicates acute depletion of virus particles due to infiltration of CD68+ and CD163+ monocytes, microglia and macrophages (Fulci et al., 2006). Depletion of these cell populations in intracranial gliomas through systemic delivery of CPA decreased intratumoral infiltration resulting in increased hrR3 titres, viral persistence within the tumor and prolonged survival (Fulci et al., 2007). Alternatively, viruses which spread by intracellular methods evade virus-specific antibodies and the complement system. HSV-1 vectors expressing the fusogenic membrane glycoprotein (FMG) have shown that the formation of multinucleated syncytia can aid in viral spread and enhance the antitumor response (Galanis et al., 2001; Nakamori et al., 2003).

In a seemingly contradictory paradigm, immunostimulatory vectors can augment the efficacy of oncolytic virotherapy by inducing an immune response against the virus as well as the tumor. It has been well established that induction of an antitumor immune response is vital to achieving complete and lasting tumor regression. The HSV-1 vector G47Δ contains a deletion in the ICP47 gene, which functions to block antigen presentation via MHC class I molecules. Accordingly, tumor cells infected with G47Δ show enhanced stimulation of tumor-associated T cells due to increased expression of MHC class I molecules compared to cells infected with G207 which expresses functional ICP47 (Todo et al., 2001b). In an immunocompetent model of neuroblastoma, G47Δ treatment enhanced tumor regression and prolonged survival compared to G207, which elicited tumor regression but did not significantly affect survival (Todo et al., 2001b). Furthermore, a combination therapy trial involving co-administration of G47Δ with immature DCs resulted in significant reductions in tumor volume and increased survival attributed to substantial lymphocyte infiltration and robust antitumor immunity (Farrell et al., 2008). The expression of peptides derived from the epitopes of tumor-associated antigens (TAAs) has been incorporated into replication-defective vectors to amplify cytotoxic T lymphocyte (CTL) responses (Rosenberg et al., 2004). However, the antitumor effects of these vectors are meagre due to immune tolerance which inhibits efficacy of the CTL response (Rosenberg et al., 2004; Frey et al., 2006). Replication-competent vectors expressing immunogenic model antigens circumvent tolerance and allow for sustained CTL responses against the bulk of the tumor due to virus spread. In a dual vaccination strategy Zhang et al. (2009) injected murine ovarian surface epithelial carcinomas (MOSECs) with vaccinia virus expressing ovalbumin (VV-OVA) followed by Semliki Forest Virus also expressing ovalbumin (SFV-OVA), or vice versa. Immune tolerance was not established and an enhanced OVA-specific CTL response and antitumor effect was observed in comparison to treatment with the wild type viruses. This demonstrates the efficacy of the bimodal approach of oncolytic virotherapy, including viral oncolysis and tumor-specific immune responses.

4.4 Safety

Concerns regarding the use of oncolytic HSV vectors in cancer therapy focus on the risk of unwanted replication in normal healthy tissue. HSV-1 is able to infect a wide variety of cell

types due to the ubiquitous expression of its cellular receptor in multiple human tissues. This presents a danger for wide-spread replication, especially when patients receiving oncolytic virotherapy are most likely immunosuppressed and highly susceptible to viral infection. Therefore, mechanisms to ensure accurate viral targeting and methods to control unwanted replication are required. Use of tissue and tumor-specific promoters, or cellular receptor-mediated targeting can be used to direct tumor-specific infection. Furthermore, Tet-off, Cre-recombinase, HSV-tk/gancyclovir regulation systems, and incorporation of suicide genes into oncolytic vectors provide a safeguard to restrict unwanted replication, should it occur. Safety evaluations in preclinical studies should include careful considerations of dose limiting toxicity, potential of reversion or acquisition of virulent phenotypes, altered tropism, and the effect of transgene expression on the host. Choosing an appropriate animal model is imperative to accurately evaluate vectors for clinical use.

Results from clinical trials using oncolytic HSV-1 vectors are promising. Adverse effects are non-life threatening, including moderate to mild flu-like symptoms and injection site irritation demonstrating the safety profile of this treatment modality. In many cases complete responses were observed resulting in prolonged patient survival and therefore, initiation of subsequent clinical trials. Oncolytic vectors, especially HSV-1-based, are rookies in the field of cancer therapy. However, with careful coaching they have the potential to achieve all-star status.

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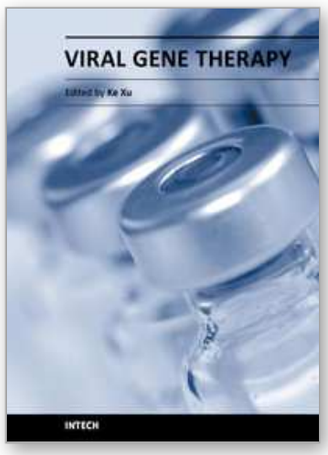

Viral Gene Therapy

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The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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