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Novel Oncolytic Herpesviruses for Breast Cancer Treatment

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NOVEL ONCOLYTIC HERPESVIRUSES FOR BREAST CANCER TREATMENT

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ABSTRACT

Oncolytic herpes simplex virus type-1 (HSV-1) has shown great potential as an effective agent for cancer therapy – oncolytic virotherapy. To enhance oncolytic capabilities of previously reported agents, new oncolytic and fusogenic HSV-1 OncSyn and OncdSyn viruses were constructed based on wild type HSV-1 (F) strain. To provide for safety and tumor selectivity, the viruses carried a large deletion including one of the two $\gamma_1$34.5 genes. The $\gamma_1$34.5 gene, a major neurovirulence factor, was replaced by a gene cassette constitutively expressing the red fluorescent protein. Homologous recombination was utilized to transfer the fusogenic gBsyn3 mutation to the viral genome to produce the OncSyn virus. The syncytial mutation gKsyn1 was introduced into the OncSyn genome cloned into a bacterial artificial chromosome using double-red mutagenesis in *E. coli* to produce the OncdSyn virus carrying both syncytial mutations gBsyn3 and gKsyn1. Both viruses caused extensive virus-induced cell fusion (syncytia) and were able to infect and replicate in mammary cancer cells. A xenograft mouse model system using MDA-MB-435S-luc human breast cancer cells constitutively expressing the luciferase gene implanted within the interscapular region of animals was utilized to test the ability of the OncSyn virus to inactivate breast tumor cells in vivo. A single round of intratumoral virus injections resulted in a drastic reduction of tumor sizes ($p \leq 0.0001$) and dimunition of chemiluminscence emitted by the cancer cells ($p \leq 0.0002$). Systematic necropsy and pathological evaluation of the primary tumors revealed that the single round of injections resulted in extensive necrosis of tumor cells ($p \leq 0.0001$). Both effects were enhanced by a second round of virus injections. The oncolytic potential of both OncSyn and OncdSyn viruses was tested in a highly metastatic syngeneic mouse model system utilizing 4T1 murine mammary cancer cells. Intratumoral injections of both OncSyn and OncdSyn resulted in significant reduction of tumor sizes ($p < 0.05$) compared to controls. Virus treated mice but not controls showed a marked reduction of

metastatic foci in lungs and internal organs. These results show that the attenuated, but highly fusogenic and oncolytic HSV-1(F) virus strains OncSyn and OncdSyn may effectively treat breast tumors in vivo.

CHAPTER I

INTRODUCTION

Statement of Problem and Hypothesis

Herpes Simplex Viruses are known to be the etiologic agents responsible for many human diseases including mucocutaneous oral and genital lesions, keratoconjunctivitis, and viral encephalitis. There are two types of herpes simplex virus, type 1 and type 2 (HSV-1 and HSV-2), both belonging to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily. Herpes simplex virus type 2 is a common human pathogenic virus and is associated with sexually transmitted diseases. Herpes simplex virus type 1 (HSV-1) is also a human pathogen, but is rarely associated with genital area infection. Acute HSV-1 infection generally involves gingivo stomatitis. Chronic HSV-1 infection has also been described and is characterized by a skin keratitis. Herpes simplex virus is capable of replicating in a wide variety of tissues, including lymphocytes. As a result of its intracellular locus of replication, it is able to escape anti-HSV antibodies during the replication process. Herpes simplex virus type 1 genomic components controlling pathogenicity have been identified.

A number of attempts have been made to exploit the cytolytic properties of viruses in the treatment of cancer. Several candidate viruses are currently under human clinical investigation, including the Newcastle disease virus, adenovirus, and herpes simplex virus families (Kooby *et al.*, 1999; Reid *et al.*, 2001; Mullen and Tanabe, 2002; Ring, 2002; Varghese and Rabkin, 2002). Herpes simplex virus type 1 has many favorable features both as a delivery vector for cancer therapeutic genes and as a backbone for oncolytic viruses. HSV-1 is highly infectious, so HSV-1 vectors are efficient vehicles for the delivery of exogenous genetic materials to cells. The inherent cytotoxicity of this virus, if harnessed and made to be selective by genetic manipulations, makes this virus a good candidate for developing viral oncolytic approach.

Furthermore, its large genome size, ability to infect cells with a high degree of efficiency, and the availability of antiviral drugs that are effective in treating disseminated viral infection provide distinct advantages over other viral systems.

Many strategies have been used to create tumor-selective oncolytic viruses (Kirn *et al.*, 2001; Nemunaitis and Edelman, 2002). These strategies can be grouped into three general approaches. The first approach is to delete gene functions that are critical for efficient viral replication in normal cells but are dispensable in tumor cells (Lou, 2003). The second approach is to limit the expression of a critical viral gene to tumor tissues through the use of tumor- and/or tissue-specific promoters (Chung *et al.*, 1999; Mullen *et al.*, 2002). The third approach is to alter viral tropism through modification of surface proteins (Goins *et al.*, 2004). All these approaches have been successfully utilized alone or in combination to construct oncolytic HSV-1 viruses for cancer treatment. However, the first generation oncolytic HSV-1 vectors have shown limitations in terms of their efficacy and safety in hosts. Novel oncolytic HSV-1 vectors are needed that can address the current drawbacks of HSV-1 oncolytic virotherapy.

HSV-1 viruses that replicate exclusively in cancer cells form the basis for designing oncolytic viral strain. The main strategy includes the deletion of one or both viral $\gamma_1$34.5 genes. The $\gamma_1$34.5 enables HSV-1 to replicate in neurons and has been described as an inhibitor of cellular apoptosis and a specific neurovirulence factor (Chou *et al.*, 1990; Bolovan *et al.*, 1994; Valyi-Nagy *et al.*, 1994a; Spivack *et al.*, 1995). There are two copies of this gene in the viral genome. The deletions of $\gamma_1$34.5 genes drastically attenuate the virus and restrict viral growth to cancer cells which are known to lack intact apoptotic mechanisms. Several mutant strains have been constructed in which both copies of $\gamma_1$34.5 gene were deleted (HSV1716 virus, R3616 virus, G207, OncoVex$^{GM\text{-}CSF}$, etc.). However, preclinical studies in animal models as well as human clinical trials have revealed that viruses that have both copies of $\gamma_1$34.5 gene deleted have

a limited ability to spread within tumors, and there is a substantial reduction in infectious virus production compared to the parental wild-type strains (McKie *et al.*, 1996; Andreansky *et al.*, 1997; Kramm *et al.*, 1997). These results have led to the hypothesis that lower or conditional expression of $\gamma_1$34.5 will largely attenuate the virus, while allowing ample replication in tumor cells. Therefore, oncolytic HSV strains have been constructed having only one of the two $\gamma_1$34.5 genes deleted (Cozzi *et al.*, 2001; Bennett *et al.*, 2002; Gutermann *et al.*, 2006).

Another potential limitation is the lack of oncolytic HSV-1 to induce a significant bystander effect as a result of its antitumor activity. The bystander effect is the killing of uninfected cells after the lysis of the infected neighboring cells. An interesting novel strategy to enhance HSV-1 bystander effect is the inclusion of fusogenic glycoproteins that enable the virus to spread to uninfected cells via virus-induced cell fusion (Bateman *et al.*, 2000; Higuchi *et al.*, 2000).

HSV-1 oncolytic viruses carrying a deletion of only one copy of $\gamma_1$34.5 gene have been extensively evaluated in preclinical studies in animals as well as human clinical trials (Bennett *et al.*, 2001; Bennett *et al.*, 2002; Gutermann *et al.*, 2006; Kemeny *et al.*, 2006; Varghese *et al.*, 2007). The idea of utilizing fusogenic viruses for the oncolytic virotherapy of cancers is relatively new. Studies using HSV-1-based oncolytic viruses containing fusion-inducing syncytial mutations of unspecified origin as well as expressing cell fusion-inducing glycoproteins have been recently reported (Fu and Zhang, 2002; Fu *et al.*, 2003; Nakamori *et al.*, 2004).

The overall hypothesis of the investigations described in this dissertation has been that HSV-1 oncolytic strains that have a single $\gamma_1$34.5 gene deleted and code for synytial mutations capable of producing extensive virus-induced cell fusion will be highly efficacious in killing tumor cells both *in vitro* and *in vivo*. We chose breast cancer as our initial model system for

oncolytic virotherapy because the constructed prototypic HSV-1 OncSyn virus (OncSyn) replicated very efficiently in human breast cancer cells. Furthermore, breast cancer continues to remain a major threat worldwide. In the United States, breast cancer is the most common non-skin cancer and the second leading cause of cancer-related death in women. Obviously, the constructed viruses could be used to treat other types of cancers as has been shown for other oncolytic herpesviruses.

Statement of Research Objectives

The goal of this research was to produce enhanced HSV-1 oncolytic viruses for the treatment of breast cancer. In this study, the previously described NV1020 HSV-1 genomic deletion was combined with virus-induced cell fusion-producing syncytial mutation/mutations to engineer HSV-1 OncSyn and HSV-1 OncdSyn novel oncolytic fusogenic viruses. These viruses were extensively characterized in tissue cultue and in breast cancer mouse model systems. The specific aims of this research were:

Specific Aim I: Construction and tissue culture characterization of new prototypic oncolytic recombinant viruses HSV-1 Onc (Onc) and HSV-1 OncSyn (OncSyn) having the main genomic characteristics of the previously constructed NV1020 virus and in addition specifying a syncytial mutation in glycoprotein B (gB).

Specific Aim II: Determination of the oncolytic potential of the constructed OncSyn virus in a mouse xenograft model of breast cancer utilizing MDA-MB-435S human breast cancer cells.

Specific Aim III: Construction and tissue culture characterization of a further improved HSV-1 OncdSyn virus carrying syncytial mutations in glycoprotein B (gB) and K (gK).

Specific Aim IV: Testing and comparision of the newly constructed viruses OncSyn and OncdSyn for their ability to inactivate mammary cancer cell tumors in a mouse syngeneic model utilizing 4T1 murine mammary adenocarcinoma cells.

Overall, the salient features of our results are:

A. The constructed attenuated, fusogenic, and oncolytic HSV-1 (F) virus strain OncSyn effectively killed tumor cells both in vitro and in vivo.

B. The engineered singly and doubly syncytial virus strains OncSyn and OncdSyn, respectively, demonstated enhanced antitumor effects and inhibition of tumor metastasis to internal organs of mice.

The work is presented in individual chapters in a manuscript format with a specific title for the central theme of each chapter:

Chapter II: Effective Treatment of Human Breast Tumor in a Mouse Xenograft Model with Herpes Simplex Virus Type 1 Specifying the NV1020 Genomic Deletion and the gBsyn3 Syncytial Mutation Enabling High Viral Replication and Spread in Breast Cancer Cells.

Chapter III: NV1020-like Herpes Simplex Virus Type-1 Oncolytic and Highly Fusogenic Mutants Effectively Inhibit Primary and Metastatic Tumors in Mice.

Literature Review

Herpesviruses

History of Herpesviruses

Herpesvirus hominis, or herpes simplex virus (HSV), is one of the most common agents infecting humans of all ages. The virus occurs worldwide and produces a variety of illnesses, including mucocutaneous infections, infections of the CNS, and occasionally infections of the visceral organs. Herpes simplex virus may cause a myriad of clinical presentations. The course depends on the age of the patient, the immune status of the host, the site of infection, the person's previous immunity to autologous or heterologous viruses, and the antigenic type of the virus.

The first description of Herpes Simplex Virus (HSV) can be traced back to the ancient Greeks. Hippocrates used the term “herpes” to describe lesions that appeared to creep or crawl

along the skin (Wildy, 1973). Descriptions of lesions resembling HSV were also found on a Sumerian Tablet dated to the 3rd Millennium BC and the Ebers Papyrus, circa 1500 BC (Whitley, 2001). Herodotus is noted as the first to describe an association between the cutaneous lesions and fever caused by HSV, and Galen recognized that recurrent HSV lesions develop at the same anatomical location (Whitley, 2001).

Hippocrates was the first to describe lesions that could have been caused by HSV, but the clinical conditions caused by this virus have only been described in more detail over the past centuries. Most of the key findings relating to HSV infection and treatment have been made since the early 20th century. These range from discovering some of the mechanisms behind virus latency and reactivation, to the development of the drug acyclovir, which was the first selective inhibitor of HSV replication.

During the 18th century, Bateman accurately described the nature of HSV infection as a "restricted group of localized vesicles with a short, self-limiting course" (Bateman, 1814). The first description of the link between HSV and the genital organs did not appear until *De Morbis Venereis* was published by John Astruc, physician for King Louis XIV, in 1736, after studying the afflictions of French prostitutes (Astruc, 1736).

During the late 19th and early 20th century, human volunteers were often used to test the transmission of infectious agents, and Vidal showed that HSV was infectious by passing it from human to another (Vidal, 1873). Gruter, in a switch from human to animal studies, demonstrated that HSV could be transmitted from rabbit to rabbit, and he is widely credited with the isolation of HSV by the virology community (Gruter, 1924). In 1939, Burnett and Williams published an article describing the nature of latency, noting that HSV seems to persist for life and can be reactivated under stressful conditions to produce visible lesions (Burnet and Williams, 1939). The development of tissue culture technology was critical in the isolation of other members of

the human herpesvirus family. Between 1952 and 1956, varicella zoster virus (VZV), the causative agent of chicken pox, and cytomegalovirus (CMV) were isolated (Weller and Stoddard, 1952; Rowe *et al.*, 1956; Smith, 1956; Craig *et al.*, 1957). The eventual cultivation of lymphoblastoid tumor cells and B lymphocytes led to the isolation and study of Epstein-Barr virus (EBV) (Epstein *et al.*, 1964). In the 1990s, cultivation of T lymphocytes led to the isolation of human herpesviruses 6A, 6B, and 7 (Salahuddin *et al.*, 1986; Lopez *et al.*, 1988; Frenkel *et al.*, 1990). More recently, Representational Differential Analysis (RDA) led to the discovery of human herpesvirus 8 (Chang et al., 1994).

As in most fields of scientific research, in the 25-plus centuries that have passed since investigations into the herpes simplex virus first began, the focus of inquiry has undergone drastic changes. Over the centuries, the inquiry has progressed, from the basic classification of lesions to descriptions of disease, epidemiology of infection and, ultimately, to the molecular characterization of this virus.

Herpes simplex virus is one of the most fascinating biological organisms known to man, and in the 21st century new applications of knowledge concerning this pathogen will emerge at many levels. Ultimately, these will lead to the development of new antiviral drugs, vaccines and gene therapy. HSV research is still in its golden age. New facts continue to emerge about HSV, and manipulation of the virus is providing much information. Genetic engineering of this virus is likely to have a most significant impact on future medical therapies, which could extend to specialties beyond virology.

Taxonomy of *Herpesviridae*

Identification of the new and apparently related viruses led to a scientific desire for classification. However, it was not until 1981 that the current herpesvirus classification came into being. All herpesviruses examined to date are capable of establishing a latent infection in

their natural hosts in a specific set of cells, which varies from one virus to another. Other biological properties vary, such as the length of the reproductive cycle, and these were used as the basis of classification, before DNA sequences of the viruses were known. Members of the family *Herpesviridae* were initially classified by the Herpesvirus Study Group into three subfamilies: the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae* (Roizman *et al.*, 1973; Roizman *et al.*, 1992; Van Regenmortel *et al.*, 2000). DNA sequence data has since supported and expanded the platform on which the classification system rests. Table 1.1 shows the classification of the nine known human herpesviruses (bold type) as well as other commonly studied herpesviruses (Roizman et al., 1992; Van Regenmortel et al., 2000).

Alphaherpesvirinae were classified based on their variable host range, short reproductive cycle, rapid spread in tissue culture, efficient destruction of infected cells, and the ability to establish latent infections primarily in sensory ganglia. The subfamily consists of the genera *Simplexvirus, Varicellovirus, Marek's disease-like virus,* and *Infectious laryngotracheitis-like virus* (Roizman *et al*., 1992; Van Regenmortel *et al*., 2000).

Betaherpesvirinae were characterized by a limited host range, long reproductive cycle, and slow infection progression in tissue culture. Cells that are infected often become enlarged (cytomegalia), and the viruses can maintain latency in secretory glands, lymphoreticular cells, kidneys, and other tissues. The subfamily consists of the genera *Cytomegalovirus, Muromegalovirus,* and *Roseolovirus* (Van Regenmortel *et al*., 2000).

Gammaherpesvirinae were classified by a limited host range and ability to replicate in lymphoblastoid cells, with some viruses also causing lytic infection in some types of epithelial and fibroblastic cells. Viruses are usually specific for either B or T lymphocytes, and latent virus is frequently demonstrated in lymphoid tissue. The *Gammaherpesvirinae* subfamily consists of the genera *Lymphocryptovirus* (EBV), and *Rhadinovirus* (Van Regenmortel *et al*., 2000).

Table 1.1: Members of the family herpesviridae

Subfamily	Designation	Vernacular Name
Alphaherpesvirinae	***Human herpesvirus 1 (HHV-1)***	**Herpes simplex virus type 1 (HSV-1)**
	Human herpesvirus 2 (HHV-2)	**Herpes simplex virus type 2 (HSV-2)**
	Human herpesvirus 3 (HHV-3)	**Varicella-zoster virus (VZV)**
	Cercopithecine herpesvirus 1 (CeHV-1)	Herpesvirus B, Simian Herpesvirus
	Gallid herpesvirus 1 (GaHV-1)	Infectious laryngotracheitis virus
	Gallid herpesvirus 2 (GaHV-2)	Marek's disease herpesvirus 2
	Suid herpesvirus 1 (SuHV-1)	Pseudorabies virus, Aujesky's disease
	Felid herpesvirus 1 (FeHV-1)	Feline herpesvirus 1, Feline rhinotracheitis herpesvirus
	Ictalurid herpesvirus 1 (IcHV-1)	Channel catfish herpesvirus
Betaherpesvirinae	***Human herpesvirus 5 (HHV-5)***	**Cytomegalovirus (CMV)**
	Cercopithecine herpesvirus 8 (CeHV-8)	Rhesus monkey cytomegalovirus
	Murid herpesvirus 1 (MuHV-1)	Mouse cytomegalovirus
	Murid herpesvirus 2 (MuHV-2)	Rat cytomegalovirus
	Suid herpesvirus 2 (SuHV-1)	Pig cytomegalovirus
	Felid herpesvirus 2 (FeHV-1)	Cat cytomegalovirus
	Human herpesvirus 6A (HHV-6A)	
	Human herpesvirus 6B (HHV-6B)	**Roseolovirus**
	Human herpesvirus 7 (HHV-7)	
Gammaherpesvirinae	***Human herpesvirus 4 (HHV-4)***	**Epstein-Barr virus (EBV)**
	Human herpesvirus 8 (HHV-8)	**Karposi's sarcoma-associated herpesvirus (KSHV)**

Clinical Significance of Herpes Simplex Viruses

Epidemiology

Infections caused by HSV occur worldwide in both developed countries and underdeveloped countries (Black, 1975). There are no known animal carriers for HSV; therefore, humans remain solely responsible for transmitting virus to other humans. Virus transmission from an infected to a susceptible individual occurs during close personal contact. The frequency of person to person contact appears to be the major mediator of infections (Whitley, 2001). Due to the fact that HSV infection rarely results in fatality and the nature of latency, more than half of the world's population probably has a recurring HSV infection, enabling the transmission of HSV. Initial HSV infection usually occurs in children less than 5

years old and is most often asymptomatic. The mouth area is the most common location of infection (Whitley, 2001). Primary infection leads to the shedding of virus from the mouth and stool for an average of 7 to 10 days (Amir *et al*., 1997), and neutralizing antibodies appear between 4 and 7 days after the onset of HSV infection and peak at approximately 3 weeks post infection (Buddingh *et al*., 1953).

The primary factors affecting the rate of HSV infection are location, socioeconomic status, and age. In some studies, by the age of 15, over 95% of children were found to possess antibodies to HSV (Black *et al.*, 1974; Bader *et al.*, 1978). HSV-1 seroprevalence is much more common than its counterpart HSV-2 (Nahmias *et al.*, 1990; Fleming *et al.*, 1997). Because HSV-2 is usually acquired through sexual contact, antibodies to HSV-2 are rarely found before the onset of sexual activity. While most genital infections are caused by HSV-2, there is an ever-increasing proportion attributable to HSV-1 (Kalinyak *et al.*, 1977; Corey *et al.*, 1983). Genital HSV-1 infections are usually both less severe than HSV-2 and less prone to recurrence (Reeves *et al.*, 1981; Corey *et al.*, 1983).

Pathogenesis

Initially, virus must come in contact with a mucosal surface or abraded skin for HSV infection to be initiated. After primary infection, viral replication at the infected location, usually oral or genital mucosal tissue, results in the infection of sensory nerve endings; and virus is then transported to the dorsal root ganglia (Bastian *et al.*, 1972; Baringer and Swoveland, 1973). In HSV-1 infection, the trigeminal ganglia becomes colonized and harbors latent virus; whereas in HSV-2 infection the sacral ganglia is the site of latency (Whitley, 2001). After the establishment of latency, certain stimuli can cause reactivation to occur, and virus becomes evident at mucocutaneous sites as vesicles or ulcers. A more severe primary infection can result in a higher rate of HSV reactivation.

Cellular changes induced by viral infection include enlargement of infected cells and the appearance of condensed chromatin within the nuclei, followed by degradation of the nuclei. Cells lose intact plasma membranes and form multinucleated giant cells. In infected dermal regions, there is an intense inflammatory response, and the intensity decreases substantially with recurrent disease (Whitley, 2001).

Mucocutaneous Infections: Primary HSV-1 infection can be either totally asymptomatic or can result in a symptoms in any combination of fever, sore throat, vesicular or ulcerative lesions. However, asymptomatic infection is generally the rule rather than the exception (Whitley, 2001). The duration of symptomatic disease in symptomatic children is generally 2 to 3 weeks, with a fever of 101°F to 104°F. The onset of a recurrent HSV-1 infection is generally marked pain, burning, tingling, or itching, which generally lasts for less than 6 hours, and is followed by vesicle formation within 24 to 48 hours (Spruance *et al.*, 1977; Spruance and Crumpacker, 1982; Spruance *et al.*, 1984). Usually, 3 to 5 vesicles appear at the border of the lip and last no longer than 48 hours. Pain is most severe at the outset of vesicle formation and resolves in 4 to 5 days. The frequency of recurrences varies greatly among individuals, and the factors that influence reactivation are poorly defined but may include fever, stress, and exposure to UV light (Segal *et al.*, 1974; Ship *et al.*, 1977).

In HSV-2, the most severe clinical symptoms are encountered with primary infection, characterized by the appearance of macules and papules followed by vesicles, pustules, and ulcers. The duration of lesions and viral secretion averages about 3 weeks. Men and women experience both similar and dissimilar symptoms (Corey, 1982; Corey et al., 1983). Preexisting immunity to HSV-1 can have a beneficial effect in reducing the severity of HSV-2 primary infections (Kaufman *et al.*, 1973; Corey *et al.*, 1981; Allen and Rapp, 1982). Recurrent HSV-2 is milder than initial infection and is characterized by the appearance of 3 to 5 vesicles (Adams et

al., 1976). Symptoms usually last 7 to 10 days, and virus is shed for an average of 2 to 5 days. The biggest problem involving recurrent genital herpes is the frequency of recurrences, which varies by individual. Recurrences usually occur several times per year; and, whether symptomatic or asymptomatic, transmission of the infection to sexual partners can occur with intimate contact (Corey et al., 1983).

Fetal and Neonatal Infections: Neonatal HSV infections occur at a rate of about 1 in 3000 deliveries per year (Nahmias *et al.*, 1983; Nahmias *et al.*, 1989), and infection occurs far less frequently than genital infections in the adult population. The type of maternal genital infection at the time of delivery is directly tied to the risk of fetal infection. If the mother has a primary infection the risk of transmission is approximately 30%, while the risk for recurrent infection is 3% or less (Brown et al., 1991). The most common route of infection, 75% to 80%, is intrapartum contact of the fetus with infected maternal secretions (Whitley, 2001). The clinical symptoms of neonatal HSV infection are a reflection of the site and extent of viral replication, with infection being almost always symptomatic and often lethal. Babies with HSV infection can have disease: localized to the skin, eye, and mouth; encephalitis with or without skin involvement; or disseminated infection involving multiple organs (Nahmias et al., 1970; Whitley et al., 1981). The highest mortality rate occurs in babies with disseminated infection. In addition, frequently occurring HSV-2 cutaneous lesions defined a group at risk for neurological problems (Whitley et al., 1991).

Infection of an Immunocompromised Host: Patients who are immunocompromised due to immunotherapy, malnutrition or acquired immunodeficiency syndrome are at risk for severe HSV infections, and these patients may develop progressive disease involving the respiratory tract, esophagus, or the gastrointestinal tract (Montgomerie *et al.*, 1969; Korsager *et al.*, 1975). Recurrent HSV infection can occur in theses patients at multiple sites and healing

occurs over an average of 6 weeks (Whitley et al., 1984). The repeated treatment required for these patients can lead to viral mutants resistant to antiviral therapy.

Central Nervous System (CNS) Infections: Encephalitis caused by HSV is the most common cause of sporadic, fatal encephalitis in this country (Olson et al., 1967). Some studies estimate a rate as high as 1250 cases per year in the United States (Whitley, 2001). Encephalitis is caused when the virus spreads past the dorsal root ganglia, in which latency is usually established, to the CNS. The mechanisms responsible for this aberrant event in the virus life cycle are unclear. The manifestations of HSV encephalitis include primarily focal encephalitis along with fever, altered behavior, and localized neurological findings. There is usually evidence of a localized temporal lobe disease (Whitley et al., 1977; Whitley et al., 1981). In untreated patients, mortality exceeds 70% and only 2.5% of patients return to normal neurological function (Whitley, 2001).

Keratoconjunctivitis: There are 300,000 new cases of HSV eye infection annually, ranking second behind trauma as the cause of corneal blindness (Binder, 1977). Primary herpetic keratoconjunctivitis can occur in either a single eye or both eyes, and healing of the cornea can take as long as one month even with antiviral therapy. Recurrent HSV eye infections occur at a similar rate to HSV-1 mucocutaneous infections and most often involve only a single eye. Repeated attacks can last for weeks or months and progressive disease can result in permanent cornea damage and vision loss (Whitley, 2001).

Prevention and Treatment of HSV Infection

The two methods for control of HSV infections are antiviral therapy and prevention. Antivirals such as acyclovir and valaciclovir, which inhibit viral DNA replication, are effective in limiting the extent of HSV infection and therefore helpful in limiting spread to uninfected individuals. However, post-exposure antiviral treatment does not prevent lifelong infection of an

individual. Prevention of HSV infection is mainly achieved through avoiding contact with infectious secretions. Vaccination would be the ideal method of HSV prevention; however, to date no HSV vaccine has been clinically successful.

Architecture of the Herpes Virion

Virions of herpes viruses can vary in size from 120nm to 300nm (Roizman and Furlong, 1974), and consist of: an electron-dense core, an icosadeltahedral capsid around the core, an amorphous tegument around the capsid, and an outer envelope containing glycoprotein spikes (Roizman and Furlong, 1974). The variability in the size of herpes virions is due mainly to variability in the makeup of the tegument and the state of the envelope. A model of the virion architecture is presented below in Figure 1.1.

The Core

The core of a mature herpes virion contains the viral DNA in the form of a torus that may appear to be suspended by a proteinaceous spindle to the capsid (Falke *et al.*, 1959; Furlong *et al.*, 1972; Nazerian, 1974). The toroidal structure is 50 nm high, with an inside diameter of 18nm and an outside diameter of 70 nm. The arrangement of the viral DNA in the torus is not known.

The Capsid

The capsid is approximately 125 nm in diameter in the form of an icosadeltahedron and features 162 capsomeres, characteristic of all herpesviruses. Capsomeres are either pentons or hexons, consisting of 5 or 6 VP5 (major capsid protein) monomers, respectively. In the capsid, the pentons are located on the icosahedral 5-fold vertices, and the hexons make up the faces and edges. Hexons also contain 6 copies of VP26, attached to the upper edge of VP5 and form a continuous ring around each hexon (Zhou et al., 1995). A heterotrimeric complex known as the triplex connects the capsomeres; the triplex consists of two copies of VP23 and one copy of VP19C and acts as a sort of scaffold for the capsid (Spencer et al., 1998).

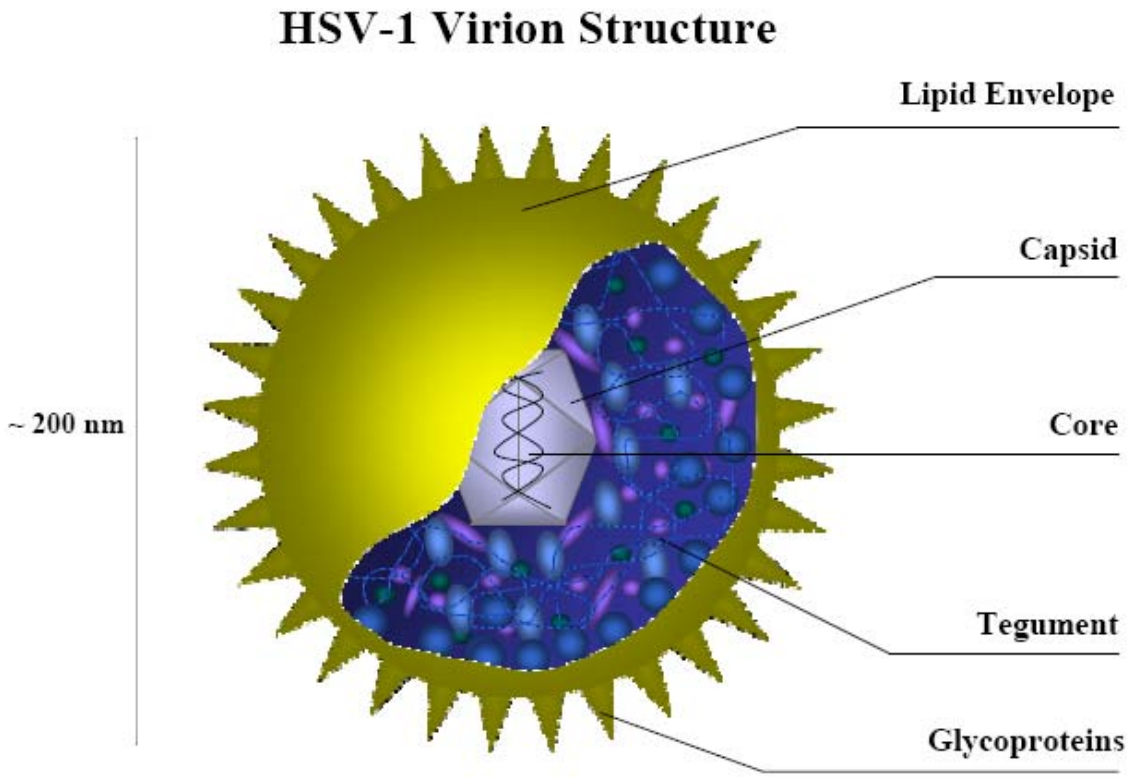

Figure 1.1: Herpesvirus virion structure. Virions of herpes viruses can vary in size from 120nm to 300nm (Roizman and Furlong, 1974). A virion consists of: an electron-dense core containing the viral genome, an icosadeltahedral capsid around the core, an amorphous tegument around the capsid, and an envelope derived from cellular membranes containing glycoprotein spikes (Roizman and Furlong, 1974).

The Tegument

The tegument is contained between the capsid and the virion envelope and appears fibrous on negative staining (Morgan *et al.*, 1959; Wildy and Watson, 1962; Morgan *et al.*, 1968). The tegument can be distributed asymmetrically and its thickness can vary depending on the location of the virion particle within the infected cell. There is less tegument that is more symmetrically arranged in peri-nuclear virions than in virions in cytoplasmic vesicles that contain more tegument distributed more asymmetrically (Falke *et al.*, 1959). Tegument proteins are important in various aspects of the virus life cycle and are believed to have key functions in the early events of infection and virion egress. There is ordered tegument density around the pentons, suggesting symmetry where the capsid and tegument interact (Zhou et al., 1999). This density may be due to the VP1-3 protein, an extremely large 336 kDa protein, thought to be involved in nucleocapsid attachment to the nuclear pore facilitating DNA release into the nucleoplasm (Knipe *et al.*, 1981; Batterson *et al.*, 1983; Ojala *et al.*, 2000). However, VP1-3 null mutants also accumulate newly assembled, DNA-filled capsids in the cytoplasm of infected cells, indicating that VP1-3 is involved in various stages of the virus life cycle (Desai, 2000).

The Envelope

The outer covering of the herpesvirus, the envelope, has a typical trilaminar appearance (Epstein, 1962) and appears to be made up of altered cellular membranes (Falke *et al.*, 1959; Armstrong *et al.*, 1961; Morgan *et al.*, 1968). The herpesvirus envelope contains numerous glycoprotein extrusions, while the amounts of each glycoprotein vary. HSV specifies at least 11 different glycoproteins, and the copy number of each glycoprotein can well exceed 1,000 per virion. Envelope glycoproteins gB, gD, gH, and gL have been shown to be required for virion entry into susceptible cells.

Organization of the Viral Genome

The viral DNA of herpesviruses is linear and double stranded, but the DNA becomes circular immediately after release from capsids into the nucleoplasm of the infected cells. The length of the genome of different herpesviruses varies between 120 to 250 kbp, with the size of HSV-1 determined to be 152,261 bp (McGeoch et al., 1988). This variability is different than polymorphism in the genome length of individual viruses, which is due to terminal and internal repeated sequences that can vary in copy number, leading to variations in genome length of more than 10 kbp. The total G+C content of herpesviruses varies from 31% to 75%, and this percentage can vary across the genome (Roizman and Pellett, 2001). HSV-1 and HSV-2 contain approximately 68% and 69% G+C content, respectively (Becker *et al.*, 1968; Kieff *et al.*, 1971).

The sequence arrangement of herpesvirus genomes varies on the presence and location of reiterated sequences that allow rearrangement to occur. In Herpes Simplex Virus genomes, the sequences from both termini are repeated in an inverted orientation and juxtaposed internally. As a result, the genome is divided into two regions, consisting of the unique long (U_L) and unique short (U_S) regions flanked by inverted repeats (Figure 1.2)

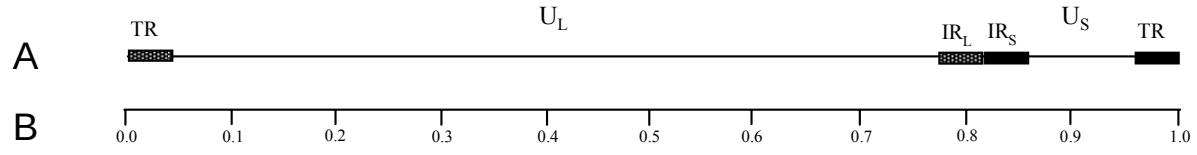

Figure 1.2: Arrangement of the HSV-1 genome. (A) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (B) The bottom line shows map units of the HSV-1 genome.

Both components are able to invert relative to the other to form four isomers; and, as predicted, DNA purified from infected cells contains four equivalent populations, which differ in the relative orientation of the unique long and unique short regions (Roizman and Pellett, 2001).

The majority of herpesvirus genes contain: a promoter region 50 to 200 bp upstream of a TATA box, a transcription initiation site 20 to 25 bp downstream of the TATA box, a 5' untranslated leader sequence of 30 to 300 bp, a single major open reading frame (ORF) with a translation initiation codon meeting the host requirement for efficient initiation, 10 to 30 bp of 3' untranslated sequence, and a polyadenylation signal with standard flanking sequences (Roizman and Pellett, 2001). Some exceptions include genes without a TATA box or genes with a second in-frame initiator methionine (Chou and Roizman, 1986; Markovitz *et al.*, 1999). Most transcriptional gene products are not spliced, although every herpesvirus expresses a few spliced genes. Herpesviruses also produce non-coding RNAs, such as the HSV-1 latency associated transcript (LAT) (Roizman and Pellett, 2001). The different members of the herpesvirus family encode between 70 and 200 genes, estimated using various methods (Roizman and Pellett, 2001). HSV-1 encodes about 90 gene products, with at least 84 of the transcriptional units encoding proteins (Roizman and Knipe, 2001).

The Herpes Simplex Virus Lifecycle

Virus Attachment and Entry

Herpesvirus entry is a multistep process involving multiple viral glycoproteins acting as ligands for multiple receptors on the surface of the target cell. Entry is the most critical step in the HSV life cycle and greatly determines the tropism and pathology of each member of the herpesvirus family. The wide host range of HSV and narrow host range of EBV can be in part explained by the ability of each virus to utilize a different array of cell surface binding and entry receptors. Entry of HSV occurs is three distinct stages: the first step involves virus binding to the

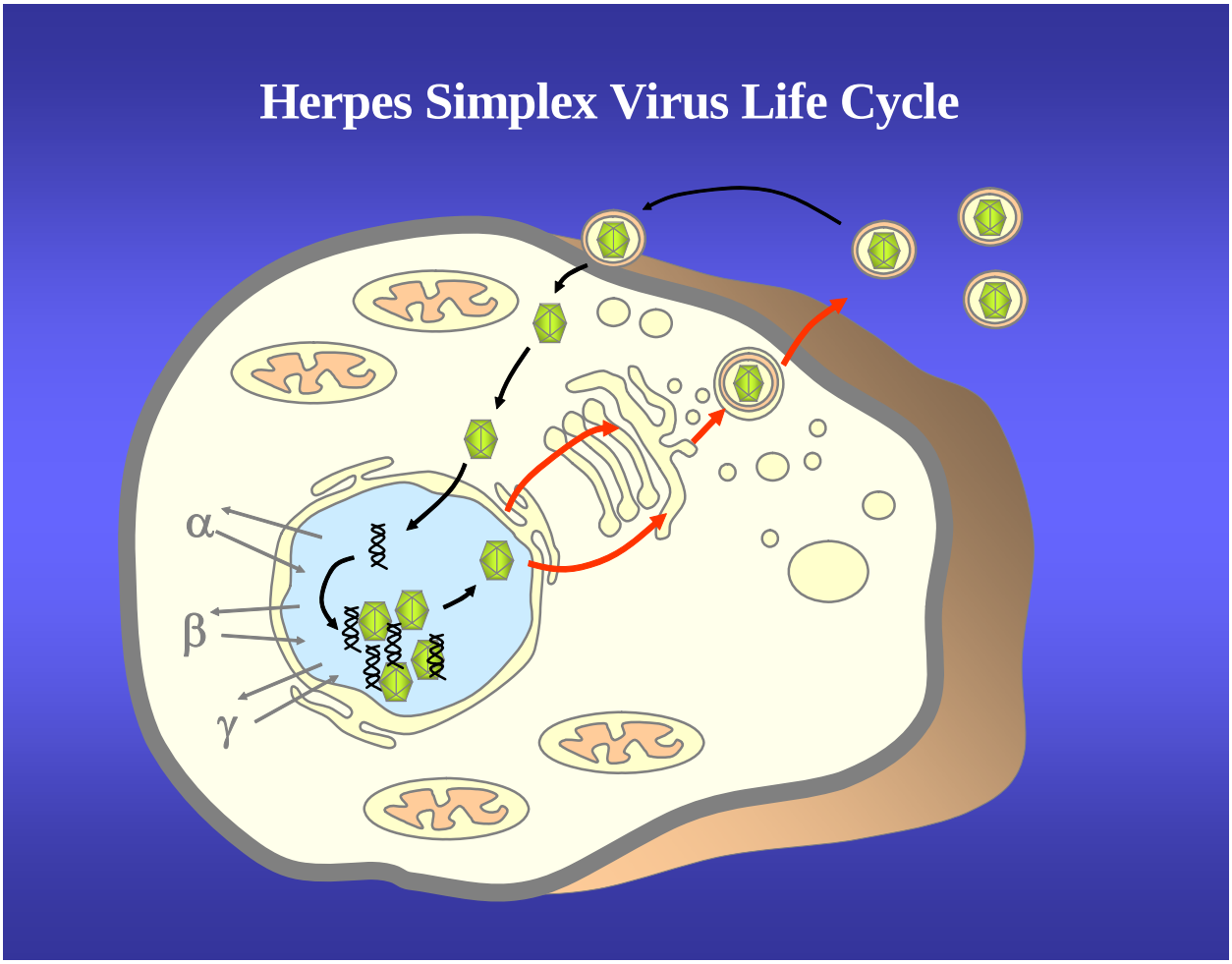

Figure 1.3: The Herpes Simplex Virus Life Cycle. The first stage of the herpes virus life cycle consists of virus entry, capsid transport to the cell nucleus, deposition of viral DNA into the nucleoplasm, coordinate gene expression and viral DNA replication (black arrows). The second stage is virion morphogenesis and egress comprised of primary envelopment at the inner nuclear membrane, de-envelopment at the outer nuclear membrane, final envelopment into cytoplasmic vesicles and transport to extra-cellular spaces (red arrows).

surface of the cell, the second step involves an interaction of gD (HSV-1) with an entry receptor, and the third step involves fusion of the viral envelope with the plasma membrane of the cell mediated by a concerted action of gH/gL and gB, releasing the capsid-tegument complex into the cytoplasm of the infected cell (Figure 1.4).

Binding Receptors

Initial contact of herpesviruses with cells usually occurs through binding receptors, in which engagement of the virus with receptor is reversible and changes in the virion envelope required for fusion and entry do not occur. Herpes simplex virus' initial attachment to cells involves the interaction of glycoprotein C, and glycoprotein B to a lesser extent, with cell surface glycosaminoglycans, usually heparan sulfate (WuDunn and Spear, 1989; Shieh *et al.*, 1992). Heparan sulfate is synthesized as the glycosaminoglycan component of heparan sulfate proteoglycans, and it is widely distributed on cell surfaces in mammals. The presence of heparan sulfate is not essential for virus entry, but it greatly increases the efficiency of virus entry into cells (Banfield et al., 1995). Similarly, although glycoprotein C confers the greatest efficiency for virus attachment to cell surfaces, increasing the efficiency of virus binding by approximately 10 fold, it is not essential for either virus entry or replication (Heine et al., 1974).

Entry Receptors

Binding of the HSV glycoprotein D to an entry receptor sets in motion an irreversible chain of events leading to the fusion of the virion envelope with the plasma membrane of the target cell. There have been three classes of HSV entry receptors classified to date, indicated below and shown in Figure 1.5.

Tumor Necrosis Factor (TNF) Receptor Family: HVEM / Herpes Virus Entry Mediator A (HveA) is a member of the TNF receptor family and is expressed in a variety of different cell types including lymphocytes, other leukocytes, epithelial cells, and fibroblasts.

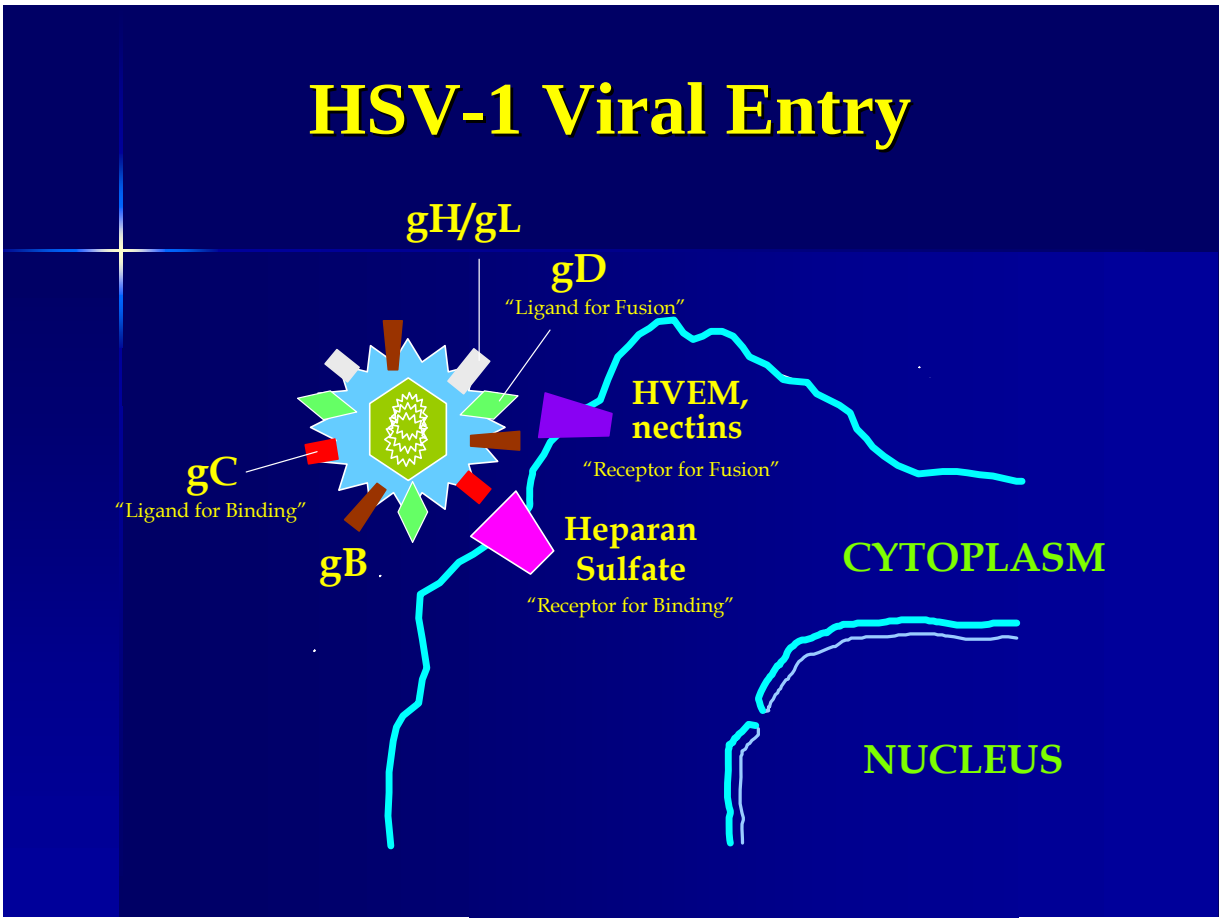

Figure 1.4: Herpes Simplex Virus Entry. The first step of HSV entry is attachment to the plasma membrane of infected cells through interaction of gC (ligand for binding) with cell surface heparan sulfate (receptor for binding). The second step is fusion of the viral envelope with the cell plasma membrane and occurs following attachment of gD (ligand for fusion) to either HVEMor a nectin (receptor for fusion) on cell surfaces. Fusion requires the presence of gD and an entry receptor, as well as gB and the gH/gL heterodimer.

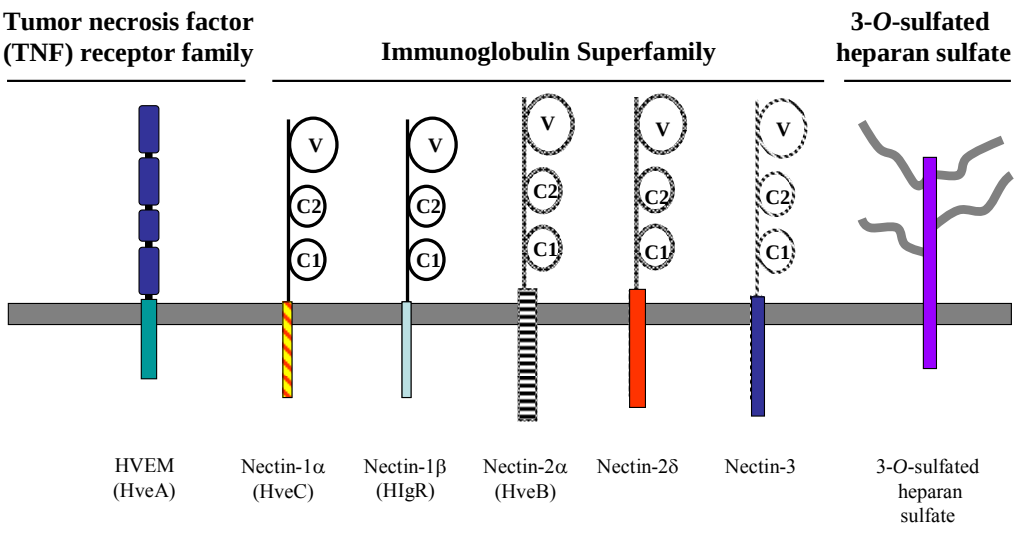

Figure 1.5: Herpes Simplex Virus entry receptors. The three classes of cell surface receptors for HSV entry are: the tumor necrosis factor (TNF) receptor family consisting of HVEM, the immunoglobulin superfamily consisting of the nectins, and 3-*O*-sulfated heparan sulfate. Only viral attachment can occur in the absence of an HSV entry receptor.

While HVEM is utilized by herpes simplex viruses as an entry receptor, the natural ligands for HVEM include LIGHT and lymphotoxin-alpha (Mauri et al., 1998). LIGHT can function as a second signal for T-cell activation, and the interactions between LIGHT and HVEM are the focus of active investigations of the regulation of immune responses (Kwon et al., 2003). HVEM serves as an excellent entry mediator for both HSV-1 and HSV-2. Certain studies have shown that HSV-1 entry of activated T-cells is principally mediated by HVEM, although in variety of other cell types HVEM was shown not to be the primary mediator of virus entry (Montgomery et al., 1996).

Immunoglobulin Superfamily: The second family of herpes simplex virus entry receptors is part of the immunoglobulin superfamily and includes several isoforms present in both human and nonhuman cells encoded by mRNAs that can vary due to alternative splicing. Members of this family have been shown to act as intercellular adhesion molecules localized at adhesion junctions, in which the carboxyl-terminal domains bind to L-afadin, a PDZ-binding

protein that anchors the receptors to the cytoskeleton and adherent junctions. These receptors have been named "nectins" on account of their cellular function (Takahashi et al., 1999).

Nectins are highly conserved among mammalian species in respect to structure, function, and the ability to mediate HSV entry (Shukla *et al.*, 2000; Milne *et al.*, 2001). The nectins are expressed in a variety of cell types including epithelial cells, fibroblasts, and neurons (Takai and Nakanishi, 2003). In addition, the distribution of this class of receptors in human tissues reflects the susceptibility of cells to infection and probably accounts for both virus entry and cell-to-cell spread (Roizman and Knipe, 2001).

Nectin-1α (Herpes Virus Entry Mediator C, HveC) and nectin-1β (Herpesvirus Immunoglobulin-like receptor, HIgR) are two mRNA splicing variants containing a common ectodomain and are expressed on epithelial, fibroblastic, neural and hematopoietic cells; in keratinocytes; and in human tissues that are the target of HSV infection including skin, brain, and spinal ganglia (Cocchi et al., 1998; Geraghty et al., 1998). Nectin-1α and nectin-1β have the ability to mediate entry of all HSV-1 strains, HSV-2, Pseudorabies virus (PrV) and bovine herpes virus 1 (BHV-1) (Geraghty et al., 1998).

Nectin-2α (Herpes Virus Entry Mediator B, HveB) and nectin-2δ are also mRNA splice variants, and they mediate the entry of HSV-2, PrV, and certain viable mutant forms of HSV-1 but not wild-type HSV-1 (Warner *et al.*, 1998; Lopez *et al.*, 2000). Nectin-1 and nectin-2 are related to nectin-3 and nectin-4, and the poliovirus receptor (Takai and Nakanishi, 2003).

Nectin-3 has been shown to be expressed on J cells, which lack HVEM, nectin-1, and nectin-2. An HSV-1 mutant, designated HSV-1 (JMP), was able to enter into J cells expressing nectin-3, but wild-type HSV-1 was not (Cocchi et al., 2004). HSV-1 (JMP) contains mutations in glycoprotein K as well as glycoprotein D. The mutations in both gK and gD were shown to be required for HSV-1 (JMP) entry into J cells (Cocchi et al., 2004).

3-O-sulfated Heparan Sulfate (3-OS HS): Modification of heparan sulfate by D-glucosaminyl 3-*O*-sulfotransferase isoforms-3 and/or -5 creates 3-*O*-sulfated Heparan Sulfate (3-OS HS), generating a gD-binding site that allows heparan sulfate proteoglycans to function as entry receptors for HSV-1. 3-*O*-sulfated heparan sulfates are broadly distributed on human cells and tissues and mediate HSV-1 but not HSV-2 entry (Shukla et al., 1999).

Other Herpesvirus Receptors: Only alphaherpesviruses (except VZV) encode members of the gD family. Betaherpesvirus and gammaherpesviruses rely on other viral proteins to serve as ligands for cellular receptors. EBV uses gp42, which becomes a member of a gH-gL-gp42 complex, to serve as a ligand for human leukocyte antigen (HLA) class II molecules on B lymphocytes. Human CMV encodes glycoprotein O (gO), which is unrelated to gp42 but also forms a complex with gH-gL (Huber and Compton, 1998). In addition, HHV-6A encodes glycoprotein Q (gQ), which forms a complex with gH-gL (Mori et al., 2003). However, it is unclear whether or not gO or gQ are required for entry in CMV or HHV-6A, respectively.

Virus-to-Cell Fusion

After binding of gD to a HSV-1 virus entry receptor, the last step in virus entry is fusion of the virion envelope with the plasma membrane of the target cell (Morgan *et al.*, 1968). The current belief is that gD (Ligas and Johnson, 1988), gB (Sarmiento *et al.*, 1979), and the gH/gL heterodimer (Forrester et al., 1992) are required for the virus-to-cell fusion process to occur. Since gD is required for entry receptor binding, it would be logical to hypothesize that receptor binding triggers a conformational change in gD that translates to a change in gB and gH/gL, resulting in activation of the HSV-1 membrane fusion machinery. In some cells types, notably CHO cells that express an entry receptor such as nectin-1 and HeLa cells, endocytosis and acidification of endosomes are required for efficient virus entry. Interestingly, it was found that endocytic uptake of HSV virions from the cell surface is rapid and independent of any known gD

receptor. In addition, efficient entry through endocytosis requires cellular PI 3-kinase activity and the viral glycoproteins gB, gD, and gH/gL (Nicola *et al.*, 2003; Nicola and Straus, 2004). While these two entry pathways are spatially distinct, they are still functionally similar in that both require the same set of viral glycoproteins and a gD entry receptor.

Recent studies involving the HSV-1 fusion machinery indicate that these glycoproteins act in a sequential manner, with gD being involved in Phase I, gH/L in Phase II, and gB in Phase III (Gianni *et al.*, 2006; Subramanian and Geraghty, 2007). Additionally, the crystal structure of HSV-1 gB has been solved, revealing a remarkable homology to vesicular stomatitis virus (VSV) gB. Along with the similarity to VSV gB, two domains that indicate HSV-1 gB is intricately involved in virus cell fusion were identified: an alpha-helical coiled-coil core reminiscent of class I fusion proteins, and two extended beta hairpins with hydrophobic tips which are indicative of class II fusion proteins (Heldwein et al., 2006). Subsequent mutagenesis studies on HSV-1 gB showed that hydrophobic tips of the loops were vital for proper HSV-1 gB function (Hannah et al., 2007).

Host Protein Shutoff

HSV shuts off the synthesis of cellular protein products in two stages. First, the virus mediates degradation of the host cell mRNA. Second, there is inhibition of further synthesis and processing of host mRNA. The virion host shutoff (*vhs*) function involves structural components of the virion and does not require de novo protein synthesis after infection (Nishioka and Silverstein, 1977; Fenwick and Walker, 1978; Nishioka and Silverstein, 1978a, b). The isolation of *vhs*$^-$ mutants, which failed to shutoff host cell protein synthesis in HSV-1 infected cells, allowed for more detailed study of the viral requirements for host shutoff (Read and Frenkel, 1983). The UL41 ORF was identified as being responsible for the phenotype in the *vhs*$^-$ mutants (Fenwick *et al.*, 1979; Oroskar and Read, 1987; Kwong *et al.*, 1988). The UL41 protein (VHS)

is expressed as a γ_1 gene (Frink *et al.*, 1981) and is translated to a 58,000 or 59,500 kDa phosphoprotein (Read *et al.*, 1993). In addition, VHS was conclusively shown to be capable of functioning in the absence of any other viral proteins (Zelus *et al.*, 1996). VHS was shown to function at least in part by inducing endoribonucleolytic cleavage of mRNA *in vitro* (Elgadi and Smiley, 1999), and the 5' end of mRNA is degraded before the 3' end (Karr and Read, 1999). VHS appears to form a complex with the transcription factor eIF-4H, and the interaction is required for RNase activity possibly through targeting to polyribosomes. The VHS and eIF-4H complex appears to decap cellular mRNA from the 5' end (Roizman and Knipe, 2001).

In the context of a *vhs*$^-$ mutant, host protein synthesis is not shut off early in infection and α and β gene expression is extended in comparison to a wild-type virus. These effects are due to the fact that VHS accelerates the degradation of both cellular and viral mRNAs. As a result, during the context of a HSV-1 infection, cellular mRNA levels diminish and as viral mRNA levels accumulate it is preferentially translated. In this way, VHS may facilitate the transition from α to β to γ gene expression through shortening the life of the viral mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1987). As a γ_1 gene product, VHS accumulates late in infection, but does not degrade viral mRNA as would be expected. An interaction between VP16 and VHS at late times blocks the degradation of RNA by VHS (Lam et al., 1996). As a result, the accumulation of VHS into tegument complexes accomplishes two crucial functions: it brings VHS into the infected cell to shutoff host protein synthesis, and it regulates the activity of VHS late in infection when mostly viral mRNA is present.

Virion Transport to the Nucleus

After fusion of the virion envelope with the plasma membrane of the infected cell, the capsid with its associated tegument complex is deposited into the cytoplasm. Some tegument proteins disassociate from the complex and remain in the cytoplasm, while others, such as VP16

(αTIF) are transported to the nucleus to effect their functions. The remaining capsid-tegument complex is then transported along the cell's microtubule network to a nuclear pore. Studies have shown that dynein, the microtubule dependent motor, is bound to capsids after entry (Sodeik *et al.*, 1997), and these results prompted the theory that incoming capsids bind to microtubules and utilize the cell's dynein motor to transport them to nuclear pores. Other experiments have shown that microtubules in proximity to the plasma membrane become disrupted after entry, suggesting that the presence of the capsid-tegument complex may destroy normal cellular microtubule interactions (Ward et al., 1998). After intracytoplasmic transport to the nucleus, capsids accumulate at the nuclear envelope and become associated with nuclear pore complexes. Experiments have shown that the VP1/2 gene is required for DNA release at the nuclear pore (Knipe *et al.*, 1979; Batterson *et al.*, 1983), and antibodies to nuclear pore transport components block capsid binding (Ojala et al., 2000). It is believed that binding to the nuclear pore complex produces a structural change in the HSV capsid, resulting in viral DNA release into the nucleus and an empty capsid docked at the nuclear pore.

Coordinate Gene Expression

During the course of an HSV infection, there are more than 80 gene products expressed from the viral genome in a highly regulated cascade fashion (Figure 1.6) in a number of coordinately expressed groups (Honess and Roizman, 1974). Transcription of viral DNA takes place in the nucleus, and the host RNA polymerase II is responsible for the transcription of all viral genes during infection (Alwine *et al.*, 1974; Costanzo *et al.*, 1977). There are several viral genes that play an important role in the regulation of gene expression at different times post-infection.

HSV encodes a function responsible for transactivation of α genes immediately after infection (Post *et al.*, 1981), and this was termed the α gene transactivating factor (α-TIF).

α-TIF was shown to be a component of the tegument capable of inducing the α genes, which contain the "TAATGArATT" promoter response element, which binds Oct-1. VP16 was identified as the viral protein responsible for the α-TIF function (Campbell *et al.*, 1984). After entry, VP16 is released from its interaction with VHS and the tegument complex. VP16 then binds to a cellular protein called the host cell factor (HCF) or C1 (Katan et al., 1990; Kristie and Sharp, 1990), and HCF carries VP16 into the nucleus, at which time the VP16-HCF complex binds to Oct-1 that is bound to viral DNA. This event forms the activator complex that is responsible for transactivation of α genes (La Boissiere *et al.*, 1999). In addition to the Oct-1 binding sites, the promoters for α genes also contain binding sites for other cellular transcriptional activators upstream of a TATA box that may contribute to a basal level of α gene expression in rapidly dividing cells in the absence of VP16 (Roizman and Knipe, 2001). The viral requirement for VP16 may be different in resting cells, such as neuronal cells, where cellular transcription factors are not available. After being deposited into the nucleus of the infected cell, the HSV-1 viral genome localizes to nuclear ND10 structures where the transcription of α genes takes place (Maul *et al.*, 1996). At 2 to 4 hours post infection, the viral α genes are expressed at peak levels. There are six viral α genes: ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$. Five of the six α genes stimulate viral β gene expression in at least some cell types. In particular, ICP4 is required for all post-α gene expression (Clements *et al.*, 1977; Dixon and Schaffer, 1980), and its effect is exerted at the transcriptional level (Godowski and Knipe, 1986). However, the mechanism by which ICP4 exerts its transcriptional control over β gene expression is unclear. ICP4 is also responsible for down regulation of α gene products including itself and ICP0, and the "pre α" gene products ORF P and ORF O. In this case, specific consensus binding sites appear to be responsible for ICP4 mediated transcriptional

regulation (Kristie and Roizman, 1984; Faber and Wilcox, 1986; Kristie and Roizman, 1986; Gelman and Silverstein, 1987; Muller, 1987). In addition, ICP4 has different isoforms dependent on different post translational modifications, and it is possible that the different functions may be somewhat dependent on the specific isoform present at different times during the course of the infection. ICP0 promotes viral infection and gene expression, especially at a low multiplicity of infection (MOI) where its absence leads to a virus yield that is 100 fold less than a wild type virus (Stow and Stow, 1986; Sinclair *et al.*, 1994). ICP0 is a nonspecific transactivator that induces the expression of the HSV α, β, and γ genes. Because ICP0 does not bind DNA directly, it appears to act indirectly in modulation of transcription (Everett *et al.*, 1991).

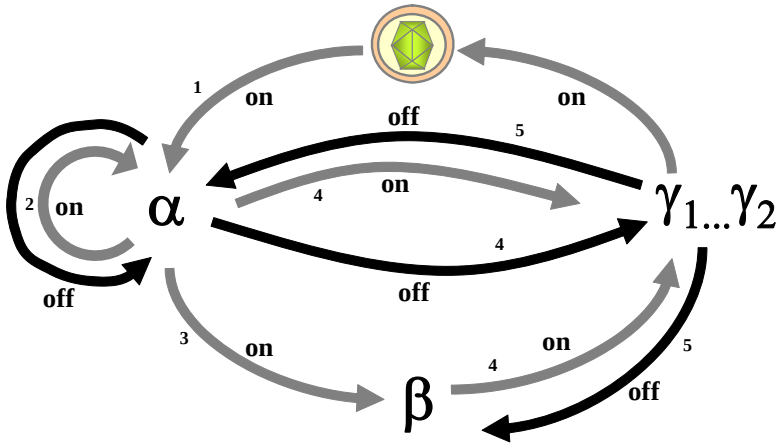

Figure 1.6: Coordinate gene expression in Herpes Simplex Virus. 1) α-TIF, a γ gene present in the tegument, activates initial transcription of the α genes. 2) Autoregulation of gene expression. 3) Activation of β gene expression. 4) Activation of γ gene expression by α and β genes, release of repression of γ genes, and replication of the viral genome. 5) γ genes turn off α and β genes late in infection (Roizman and Knipe, 2001).

The HSV-1 β genes are produced at peak levels between 4 and 8 hours post infection. Expression of the viral β genes requires the presence of functional ICP4, but is not dependent on viral DNA synthesis. β gene products include proteins involved in viral DNA replication and nucleotide metabolism. These viral proteins promote viral DNA replication, which results in expression of the γ class of genes. The β genes can be divided into two general groups: β_1 genes, which are expressed shortly after the synthesis of α proteins; and β_2 genes, which are expressed with more of a delay after a gene expression (Roizman and Knipe, 2001). β_1 genes are exemplified by the single-stranded DNA binding protein, ICP8, and the large subunit of ribonucleotide reductase, ICP6. β_2 genes are exemplified by the viral thymidine kinase encoded by UL23. Some β_2 genes require ICP27 for expression, and this dependence may correlate with the later expression of these genes (Roizman and Knipe, 2001).

The HSV-1 γ (late) genes are produced at peak levels only after viral DNA replication has started, and require ICP4, ICP27 and ICP8 for efficient levels of transcription. The transition from β to γ gene expression is also marked by a change in the nuclear localization of transcription from sites near ND10 domains to replication compartments as evidenced by the localization of ICP4, RNA polymerase II, and ICP22 (Knipe *et al.*, 1987; Rice *et al.*, 1994; Leopardi *et al.*, 1997). γ gene products include structural proteins, glycoproteins, and tegument components required to prepare newly infected cells for an efficient infection. The γ genes have been subdivided into two groups based on timing of expression and their dependence on viral DNA replication: γ_1 (leaky-late) genes, which are expressed relatively early in infection and transcription is increased only a few fold after DNA replication has occurred; and γ_2 genes, which do not accumulate in appreciable amounts until after DNA replication and are not expressed in the presence of inhibitors of viral DNA synthesis (Wagner, 1985). Typical γ_1 genes

include the major capsid protein ICP5, gB, gD, and ICP34.5, whereas typical γ_2 genes include gC, UL41 (VHS), UL36, UL38, UL20, and gK.

Viral DNA Replication

After the β genes have been expressed and translated, there are several proteins that are localized to the nucleus where they assemble on the parental viral DNA in punctuate "pre-replicative sites" located near nuclear ND10 structures (Ishov and Maul, 1996; Uprichard and Knipe, 1996). Initially, viral DNA replication initiates on the circular viral DNA, creating a "theta" structure, which as replication progresses transitions to a rolling circle mechanism of replication producing head-to-tail concatemers of viral DNA (Jacob *et al.*, 1979). At this point, replication takes place in "replication compartments" that consist of accumulating DNA molecules and replication complexes (Quinlan *et al.*, 1984).

There are seven viral proteins absolutely required for viral DNA replication and sufficient to replicate a viral origin transfected into cells. These are the viral DNA polymerase (UL30) (Purifoy *et al.*, 1977), its accessory protein (UL42) (Conley et al., 1981), an origin-binding protein (UL9), the single stranded DNA binding protein (ICP8), and the helicase-primase complex that consists of three proteins: UL5, UL8, and UL52 (Challberg, 1986; Wu et al., 1988). Host cell factors may also be involved in DNA synthesis, and host enzymes that include the DNA polymerase α-primase, DNA ligase, and topoisomerase II are almost certainly also required. There are also three origins of replication located on the viral genome: *oriS*, a palindromic sequence of 45 bp that is located in c sequences bounding the S component and present in two copies; and *oriL*, a palindromic sequence of 144 bp that is located between the transcriptional units of ICP8 and the DNA polymerase (UL29 and UL30, respectively) (Frenkel *et al.*, 1976; Locker *et al.*, 1982; Mocarski and Roizman, 1982; Stow, 1982; Vlazny *et al.*, 1982; Weller *et al.*, 1985; Knopf, 1986; Lockshon and Galloway, 1986; Deb and Doelberg, 1988). The

reason for the presence of three origins of replication is not clear, although it may reflect the evolutionary history of the virus. Only one origin of replication is needed for replication to occur (Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993).

HSV viral DNA replication proceeds as follows. First, the parental viral DNA is circularized upon being deposited into the nucleus of the infected cell. After α and β gene expression, UL9 binds to specific elements in either *oriL* or *oriS* and begins to unwind the viral DNA. UL9 then recruits the ssDNA binding protein ICP8 to the unwound portion of the viral DNA. At this point, UL9 and ICP8 recruit the remaining five proteins to the replication forks. The helicase–primase and viral DNA polymerase complexes assemble at each replication fork and initiate theta form replication. Through an unknown mechanism, replication switches from theta form to the rolling circle form of replication, and UL9 is not required for rolling circle replication because it is not origin dependent. The rolling circle replication forms long head-to-tail concatamers of viral DNA, which become cleaved into individual units during packaging of viral DNA into capsids (Roizman and Knipe, 2001).

Capsid Assembly and Packaging

After DNA replication has started, the γ proteins are transcribed, including the HSV-1 capsid proteins. First, empty shells containing an internal scaffold are assembled, with the internal scaffold lost upon viral DNA packaging into the capsid. Then the mature capsid is able to proceed along the viral egress pathway. At least some of the initial stages of capsid assembly occur in the cytoplasm of the infected cell (Nicholson et al., 1994; Rixon et al., 1996). The major capsid protein (VP5), the outer tip of hexons (VP26), and a triplex protein (VP23) are not capable of nuclear localization on their own; however, VP5 can be carried into the nucleus by VP19C, another capsid triplex protein, or by pre-VP22a, a scaffolding protein. VP23 localizes to the nucleus only in the presence of VP19C, while VP26 localizes to the nucleus only when it is

expressed with both VP5 and VP19C or pre-VP22a (Nicholson et al., 1994; Rixon et al., 1996). Electron microscopic studies have shown that final envelopment occurs in the nucleus; and three types of capsids, called *A*, *B*, and *C* capsids have been identified by sucrose density gradient ultracentrifugation (Gibson and Roizman, 1972). All three types of capsids are about 120 nm in diameter with an outer shell composed of hexons and pentons made up of VP5. The individual capsomeres are linked by triplex structures consisting of VP19C and VP23, the two minor capsid proteins. Each triplex consists of one molecule of VP19C and two molecules of VP23 (Newcomb et al., 1993). *C*-capsids are mature capsids that contain packaged viral DNA and can proceed to become infectious virions by budding through the nuclear membrane into the peri-nuclear space (Perdue et al., 1976). In contrast, *A* and *B* capsids lack viral DNA, but *B* capsids are filled with VP22a and VP21, the cleaved scaffolding proteins, and a viral protease VP24 (Gibson and Roizman, 1972; Newcomb et al., 1993). The internal proteins VP22a, VP21, and VP24 are removed upon packaging of viral DNA to form *C* capsids (Gibson and Roizman, 1972; Davison *et al.*, 1992). *A* capsids do not contain viral DNA or scaffolding proteins and are believed to be an abortive result of failed attempts at DNA packaging.

In terms of the order of assembly, once in the nucleus, VP5-pre-VP22a complexes come together as a result of self assembly of pre-VP22a. The triplex proteins VP19C and VP23 are then added to form a partial capsid. As hexons and pentons are added, the structure assembles into a round procapsid (Newcomb et al., 1996). At this point, the procapsid undergoes a structural transformation and becomes polyhedral (Thomsen *et al.*, 1995; Trus *et al.*, 1996; Church and Wilson, 1997). It is unknown at this time whether the round or polyhedral *B* capsids are the structure that viral DNA is packaged (Perdue *et al.*, 1974; Lee *et al.*, 1988; Trus *et al.*, 1996). Encapsidation of viral DNA is a process in which unit length monomers of viral DNA are cleaved from concatamers and packaged into preformed *B* capsids, as originally shown for

pseudorabies virus (Ladin *et al.*, 1980; Ladin *et al.*, 1982). Cleavage of concatamers occurs at specific sites and requires the *pac1* and *pac2* packaging signals (Varmuza and Smiley, 1985; Deiss *et al.*, 1986; Smiley *et al.*, 1990). The process of encapsidation of viral DNA into capsids is complex and requires several gene products, including the UL6, UL15, UL25, UL28, UL32, UL33, UL36 and UL37 gene products, but the mechanism of viral DNA packaging is not well defined (Roizman and Knipe, 2001).

Herpesvirus Egress

Nuclear Egress: Primary Envelopment: After encapsidation of genomic DNA, mature nucleocapsids acquire primary envelope by budding through the inner nuclear membrane into the peri-nuclear space (Vlazny *et al.*, 1982). Two conserved herpesvirus proteins, UL31 and UL34 have been shown to be involved in the budding process. UL34 is C-terminally anchored membrane protein present in the inner and outer nuclear membrane (Klupp *et al.*, 2000a), while UL31 is a nuclear phosphoprotein also present in the nuclear membrane of infected cells (Reynolds *et al.*, 2001; Fuchs *et al.*, 2002). The UL31 protein requires UL34 for proper nuclear targeting (Fuchs et al., 2002), whereas the UL34 protein appears to possess an intrinsic nuclear targeting signal (Klupp *et al.*, 2000a). However, the nuclear targeting of UL34 is increased by the presence of UL31 (Reynolds *et al.*, 2001; Fuchs *et al.*, 2002). Analysis of UL31 and UL34 deletion mutants has indicated that the absence of either protein results in a drastic impairment in primary envelopment with capsids trapped within the nucleus (Chang *et al.*, 1997; Klupp *et al.*, 2000a; Roller *et al.*, 2000; Reynolds *et al.*, 2001; Fuchs *et al.*, 2002). In addition, partial dismantling of the nuclear lamina has been observed after HSV infection and may be required so that intranuclear capsids are able to gain access to the inner nuclear membrane (Scott and O'Hare, 2001). Multiple lines of evidence indicate that UL31 and UL34 play a role in dismantling the nuclear lamina in infected cells. Overexpression of the UL31 protein in

uninfected cells was sufficient to relocalize lamin A/C from the nuclear rim into nucleoplasmic aggregates, while over expression of UL34 was sufficient to relocalize some lamin A/C into the cytoplasm (Reynolds *et al.*, 2004). More importantly, both UL31 and UL34 have been shown to directly bind lamin A/C, suggesting that the UL31 and UL34 proteins modify the conformation of the nuclear lamina in infected cells, possibly by direct interaction with lamin A/C. Given that the nuclear lamina potentially excludes nucleocapsids from envelopment sites at the inner nuclear membrane, the lamina alteration may reflect a role of the UL31/UL34 protein complex in perturbing the lamina to promote nucleocapsid egress from the nucleus (Baines 2004). It is unclear whether additional primary envelope and tegument proteins exist that are required for primary envelopment to take place. The UL11 protein has been proposed to function in primary envelopment and increases the efficiency of envelopment at this step (Baines and Roizman, 1992; MacLean *et al.*, 1992).

Egress from the Peri-nuclear Space: De-Envelopment: The subsequent steps in HSV-1 egress following primary envelopment have been in dispute for some time. Two models were proposed to explain virion egress. The first model, sometimes called the "lumenal" pathway (CH72 144), suggests that peri-nuclear virions retain their primary envelope and integrity as they leave the cell through the secretory pathway (Darlington and Moss, 1968; Johnson and Spear, 1982; Campadelli-Fiume *et al.*, 1991; Johnson *et al.*, 2001). In this model, virion glycoproteins are modified in transit to the plasma membrane and peri-nuclear virions should contain the entire complement of tegument and envelope proteins present on mature extra-cellular virions. The alternative model proposes that the primary envelope of peri-nuclear virions fuses with the outer nuclear lamellae, resulting in loss of the primary envelope and some tegument components and translocation of the capsid into the cytoplasm of the infected cells (Figure 1.7, step II) (Enquist et al., 1998; Mettenleiter, 2000). In this "de-envelopment re-envelopment" pathway, final

tegumentation and envelopment (re-envelopment) occur in cytoplasmic compartments (Figure 1.7, step III). In addition, in this model peri-nuclear and intracytoplasmic/extra-cellular virions should differ in composition. There is evidence that supports both the "lumenal" and the alternative "de-envelopment re-envelopment" pathways. However, the vast majority of recent evidence supports the "de-envelopment re-envelopment" model of HSV-1 virus egress. The first line of evidence supporting the alternative model is that primary envelope and primary tegument of peri-nuclear virions clearly differ in ultra-structural appearance from the final envelope and final tegument of extra-cellular virions when observed by electron microscopy (Gershon et al., 1994; Granzow et al., 2001). In addition, while UL31 and UL34 localize on nuclear membranes and peri-nuclear virions, the UL31 and UL34 proteins were not detected in extra-cellular virions (Reynolds et al., 2002). In further support of the envelopment-de-envelopment model, the major tegument proteins UL46 and UL49 are present in intracytoplasmic/extra-cellular virions but absent from peri-nuclear virions (Klupp *et al.*, 2000a; Mettenleiter, 2002b). Also, the phospholipid composition on the final virion envelope differs substantially from the composition of the nuclear membrane, explained only by a two-step envelopment model of HSV virion egress (van Genderen et al., 1994). Electron microscopic analysis has also demonstrated the fusion of primary envelopes with the outer nuclear membrane with many herpesviruses including HSV-1 (Harms *et al.*, 2000; Granzow *et al.*, 2001). As a result, the two-step "de-envelopment re-envelopment" model of virion egress is congruent with both biochemical and morphological data and constitutes a unified model for the morphogenesis of herpesviruses (Mettenleiter, 2002b). The mechanism of virion de-envelopment from the peri-nuclear space is unclear. Deletion of the major glycoproteins involved in membrane fusion events does not affect the de-envelopment process as indicated by multiple studies (Cai *et al.*, 1987; Jayachandra *et al.*, 1997; Steven and Spear, 1997; Granzow *et al.*, 2001). It is possible that these glycoproteins function in a

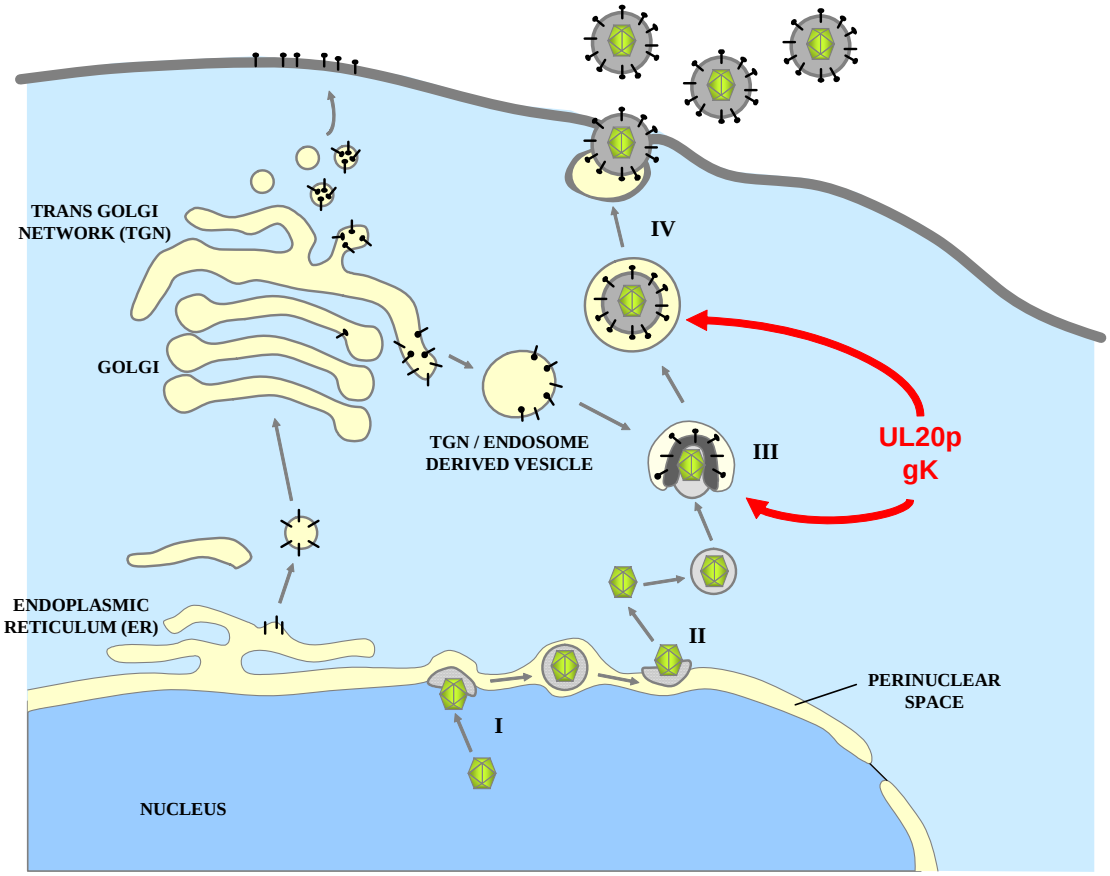

Figure 1.7: Representation of HSV-1 virion morphogenesis and egress. (I) Mature capsids budding through the inner nuclear membrane into the peri-nuclear space; (II) De-envelopment of peri-nuclear virions at the outer nuclear membrane; (III) Re-envelopment of cytoplasmic capsids by budding into cytoplasmic vesicles; (IV) Final egress to the extra-cellular space. The steps at which UL20 and gK are thought to function in virion egress are indicated by red arrows.

redundant manner, and that only deletion of multiple genes encoding glycoproteins would have an observable effect on virion de-envelopment at the outer nuclear membrane.

Tegumentation in the Cytoplasm: While the complexity of the herpesvirus capsid is not much different than that of other icosahedral viral capsids such as the picornavirus capsid, the complexity of the herpesvirus tegument is drastically increased. At least 15 proteins have been identified that are part of the HSV-1 tegument. Even more proteins make up the tegument of other herpesviruses such as VZV or CMV (Gibson, 1996; Spengler et al., 2001). Recent data indicate that tegumentation follows an intricate pattern of protein-protein interactions that contains significant levels of redundancy built in, at least as observed in cell culture (Mettenleiter, 2002b). The tegument proteins interact with the capsid on one side and the viral envelope proteins on the other side in order to link the structural components to the final envelope of the HSV-1 virion and to secure the integrity of the virus particle (Figure 1.7) (Mettenleiter, 2002b). For a long time the herpesvirus tegument was considered unstructured; however, cryoelectron microscopic analysis has indicated that at least the innermost portion of the tegument located adjacent to the capsid exhibits icosahedral symmetry, resulting from the interaction of a large tegument protein, presumably UL36, with the pentons of the capsid (Zhou et al., 1999). The UL36 gene product has been shown to interact with the major capsid protein VP5, which forms both the pentons and hexons (Machtiger et al., 1980; McNabb and Courtney, 1992; Newcomb et al., 1993). So, it appears that the first layer of tegument around the capsid is structured and composed of UL36. UL37 has been shown to interact with UL36 in co-immunoprecipitation and yeast two-hybrid experiments, and capsids that accumulate in the absence of UL37 contain UL36 (Klupp et al., 2002). UL36 and UL37 appear to be the only tegument proteins conserved in all herpesvirus subfamilies, and the absence of the HSV-1 UL36 and UL37 proteins abolishes virus maturation (Desai, 2000; Desai *et al.*, 2001; Klupp *et al.*,

2001). Unfortunately, the subsequent steps in capsid tegumentation are still largely undefined. Virion morphogenesis still proceeds in the absence of several other tegument proteins, including UL13, US3 (Purves et al., 1987), UL41, UL46, UL47 (Rafield and Knipe, 1984; Roizman and Knipe, 2001), and UL49 (Mettenleiter, 2002b). So far the most dramatic effect has been seen upon deletion of UL48 (α-TIF), which is responsible for transducing α gene promoters and makes up a major part of the virus tegument (Heine *et al.*, 1974; Batterson and Roizman, 1983). The absence of UL48 may interfere with a later step in virion assembly, presumably affecting tegumentation and re-envelopment in the cytoplasm (Mossman et al., 2000). Interestingly, UL48 has been shown to interact with other tegument components, UL49 and UL41 (VHS) (Smibert et al., 1994). Mutant forms of UL41 that do not bind UL48 do not become incorporated into the virion (Read *et al.*, 1993). Cross-linking studies have indicated that UL48 may interact with gB, gD, and gH (Zhu and Courtney, 1994), although these interactions have not been confirmed through more stringent methods.

Final Envelopment at the Trans-Golgi Network/Endosomes and Egress to Extra-cellular Spaces: Following tegumentation in the cytoplasm, HSV-1 capsids bud into cytoplasmic vesicles derived from the trans-Golgi Network (TGN) or endosomes, acquiring their final envelope (Figure 1.7, step III). Although the mechanism of secondary envelopment is not well defined, the study of mutants that contain deletions of certain glycoproteins has shed light on some requirements of the final envelopment event. By far the most dramatic effect on secondary envelopment and final egress is caused by the deletion of either UL20 or gK, and ultra-structural study of UL20-null and gK-null infected cells reveals a dramatic accumulation of un-enveloped and aberrantly enveloped capsids in the cytoplasm of infected cells with a marked absence of extra-cellular virus. With regard to gK-null and UL20-null virus titers, there is also a corresponding lack of infectious virus in the supernates of infected cells, with close to wildtype

levels of intracellular virus (Jayachandra *et al.*, 1997; Foster *et al.*, 2004a). Following the final envelopment step, nascent virions must still egress to extra-cellular spaces. The current belief is that cellular transport machinery is utilized in this step, although the mechanisms are unclear. It is apparent that the late stages in viral egress may differ depending on the cell type that is infected. For example, deletion of gE and gI, which have been shown to interact and form a complex, does not impair productive replication of HSV-1 (Enquist et al., 1998). However, in polarized epithelial cells, wild-type HSV-1 virions are sorted predominantly to cell junctions, while gE/gI null virions are non-specifically released into supernatants (Dingwell and Johnson, 1998). Delivery of virus particles to cell junctions would be expected to enhance virus spread and enable viruses to avoid host immune defenses.

Deletion of multiple genes encoding envelope glycoproteins has revealed that certain aspects of final envelopment and egress may feature redundant mechanisms, each of which is sufficient for function, at least in cell culture. For instance, while neither gE/gI or gM is required for productive replication in PrV or HSV-1, simultaneous deletion of gE/gI and gM results in drastic inhibition of plaque formation and replication in PrV (Brack et al., 1999). In addition, simultaneous deletion of UL11 and gM resulted in similar egress defect in PrV (Kopp et al., 2004). In contrast, simultaneous deletion of gE/gI and gM does not have a major effect in HSV-1 (Browne *et al.*, 2004); however, a triple mutant lacking gD, gE, and gI had a severe defect in the final envelopment step. The authors proposed that HSV gD and the gE/gI heterodimeric complex act in a redundant fashion to anchor the virion envelope onto tegument-coated capsids (Farnsworth *et al.*, 2003). In contrast, it is also possible that deletion of multiple glycoproteins indirectly disrupts the integrity of many different protein-protein interactions required for the final envelopment step and that the resulting phenotype results from the compounded effects of

an abnormal glycoprotein profile. More study of the reason for defects in double and triple null viruses is required before conclusions can be drawn regarding mechanisms of final envelopment.

Formation of Light (L) Particles: The presence of egressing capsids in the cytoplasm is not required for tegument assembly and subsequent envelopment to take place. The formation of extra-cellular herpesvirus light (L) particles that lack capsids, consist of only tegument and envelope, and are formed independent of normal virus maturation have been thoroughly studied in HSV-1 (McLauchlan and Rixon, 1992; Rixon *et al.*, 1992). L particles appear to contain the full complement of tegument proteins as well as an authentic envelope containing all appropriate glycoproteins. Studies of PrV have shown that L particle formation occurs in the absence of UL36 and UL37, which are tegument components that are critical for capsid tegumentation. It is possible that normal tegumentation may be centered on UL36 and UL37 interactions with the capsid and that UL49 may be required for proper interactions with viral glycoproteins in the final budding and envelopment steps. In the absence of capsids, tegument assembly could proceed anchored on UL49, resulting in the formation of L particles (Mettenleiter, 2002b).

HSV-1 Glycoproteins and Their Putative Functions

Glycoprotein B

The HSV-1 UL27 gene encodes the 904 aa glycoprotein B (gB). gB is highly conserved across all subfamilies of herpesviruses. HSV-1 gB is homotrimeric (aa) type I membrane glycoprotein composed of a 696-aa ectodomain that is N-glycosylated at multiple sites (Claesson-Welsh and Spear, 1986, 1987; Highlander *et al.*, 1991; Spear, 1993; Laquerre *et al.*, 1996; Whitley, 2001), a 69-aa transmembrane domain, and a 109-aa carboxyl-terminal domain. The cytoplasmic domain of gB is the longest among HSV-1 glycoproteins, implying a crucial role for this domain in gB-mediated functions. A variety of evidence indicates that gB plays important roles in membrane fusion phenomena during virus entry and virus-induced cell fusion:

HSV-1 mutant viruses lacking gB are not able to enter into cells (Cai et al., 1987) due to a post-attachment defect that can be resolved by polyethylene glycol mediated fusion of viral envelopes with cellular membranes (Cai *et al.*, 1988a); Single amino acid substitutions and truncations of the carboxyl terminus of gB cause extensive virus-induced cell fusion (Bzik *et al.*, 1984; Cai *et al.*, 1988b; Baghian *et al.*, 1993; Gage *et al.*, 1993); Transient co-expression of gB with gD, gH and gL causes cell-to-cell fusion, which is substantially increased by carboxyl terminal truncations of gB (Klupp *et al.*, 2000b; Foster *et al.*, 2001; Haan *et al.*, 2001; Pertel, 2002). Recent evidence suggests that these glycoproteins act in a sequential manner, with gD being involved in Phase I, gH/L in Phase II, and gB in Phase III (Gianni *et al.*, 2006; Subramanian and Geraghty, 2007). The crystal structure of HSV-1 gB has been recently solved, revealing a remarkable homology to vesicular stomatitis virus (VSV) gB. In addition to homology with VSV gB, two domains that indicate HSV-1 gB is intricately involved in virus cell fusion were identified: an alpha-helical coiled-coil core reminiscent of class I fusion proteins, and two extended beta hairpins with hydrophobic tips which are indicative of class II fusion proteins (Heldwein et al., 2006). Subsequent mutagenesis studies on HSV-1 gB showed that hydrophobic tips of the loops were vital for proper HSV-1 gB function (Hannah et al., 2007).

Glycoprotein C

The 1,536 bp HSV-1 UL44 gene encodes for the 511 aa long precursor of glycoprotein C (gC) (Frink et al., 1983). gC contains a 25 aa signal sequence at the N-terminus, a long 453 aa extra-cellular domain, a 23 aa transmembrane anchoring domain, and a short 10 aa C-terminal cytoplasmic tail (Homa et al., 1986). The first contact of virions with the surface of susceptible cells is through glycosaminoglycans (GAGs). gC can mediate the initial binding of HSV-1 virus to a cell surface GAG, heparan sulfate (HS). The heparan sulfate binding site of gC has been localized to the N-terminal 120 aa (Tal-Singer et al., 1995). The aminoglycoside neomycin

interferes with the binding of virus to HS mediated by gC (Herold et al., 1994). Although gC is dispensable for the infection of cultured cells, its presence can increase the efficiency of virus binding almost 10-fold, at least for HSV-1. The main function of gC binding to GAGs seems to be concentration of the virus on cell surfaces, enabling the more stable interaction of gD with an entry receptor.

Another important function of gC is its ability to inactivate complement to facilitate immune evasion by HSV-1. The gC of many herpesviruses has the ability to bind C3b (Friedman *et al.*, 1984; Eisenberg *et al.*, 1987; Huemer *et al.*, 1993; Huemer *et al.*, 1995). The presence of gC protects the virus and infected cell from antibody (Ab)-independent complement neutralization and cell lysis (Lubinski *et al.*, 1998; Lubinski *et al.*, 1999), antibody dependant complement neutralization (Hook et al., 2006), and is an important virulence factor *in vivo* (Kotwal *et al.*, 1990; Isaacs *et al.*, 1992; Sahu *et al.*, 1998). HSV-1 mutant viruses lacking gC are rapidly inactivated by human complement, resulting in up to a 5000 fold loss of titer (Friedman et al., 1996). While Ab is not required for neutralization of a gC null virus, the presence of Ab enhances neutralization (Harris *et al.*, 1990; Friedman *et al.*, 1996).

An unresolved issue regarding gC function is the relationship of gC to virus-induced syncytia formation. Several spontaneously arising syncytial virus strains were found to be gC-deficient. In particular the MP strain variant MP10311 contains a syncytial mutation in gK and is gC-deficient (Bartoletti et al., 1985). In general, the syncytial phenotype of these strains was found to be more effectively expressed in the absence of gC, especially in Hep-2 cells (Manservigi *et al.*, 1977; Bond *et al.*, 1982; Tognon *et al.*, 1984; Goodman and Engel, 1991; Pertel and Spear, 1996). One possible explanation of the enhancement of the syncytial phenotype is that in the absence of gC, because extra-cellular virus does not bind to cell surfaces

as rapidly, virus-induced cell fusion acts as the preferential method of cell-to-cell spread of HSV-1.

Glycoprotein D

The 1182 bp long US6 gene specifies a 394 aa precursor to glycoprotein D (gD). gD consists of a 25 aa signal peptide, a long 315 aa ectodoamin containing three glycosylation sites (Watson *et al.*, 1982; McGeoch *et al.*, 1985), a 22 aa transmembrane domain, and a 32 aa C-terminal cytoplasmic domain (Minson et al., 1986). The ectodomain of gD has 6 cysteine residues at positions 66, 106, 118, 127, 189, 202 forming disulfide bonds (Long et al., 1992). Importantly, gD has been shown to act as the viral ligand for all known HSV-1 entry receptors (Spear *et al.*, 2000). As a result, gD is absolutely required for virus entry and virus-induced cell-to-cell fusion to occur. As discussed earlier, HSV-1 gD has the ability to bind HVEM, nectin-1, nectin-2, and other cell surface receptors to mediate virus entry. In addition, cells that express gD are resistant to HSV infection in a dose-dependent manner due to a saturation of the corresponding entry receptors (Campadelli-Fiume *et al.*, 1988; Johnson *et al.*, 1990). X-ray structures of HSV-1 gD alone and in complex with HVEM revealed that a portion of gD assumes an Ig-like fold with unconventional disulfide-bonding patterns (Carfi et al., 2001). There is an N-terminal extension from the Ig-like fold that forms a hairpin loop in the complex with HVEM but is disordered in the crystals of gD alone. The contacts in gD for HVEM have been localized to amino acids 7 to 15 and 24 to 32 within the N-terminal hairpin (Spear and Longnecker, 2003). Mutagenic analysis of HSV-1 gD revealed that the first 32 amino acids of the N-terminal extension have a critical role in functional interactions of gD with all the HSV entry-fusion receptors, except for nectin-1, and that the amino acid sequence within this region governs whether nectin-2 can be recognized as an entry and fusion receptor (Yoon et al., 2003; Zago and Spear, 2003).

Glycoproteins E and I

The 1652 bp long US8 gene encodes the 550 aa glycoprotein E (gE) (Lee *et al.*, 1982), while the 1172 bp long US7 gene encodes 390 aa glycoprotein I (gI) (Longnecker et al., 1987). HSV, VZV, and PrV express a heterodimer of gE and gI that functions to mediate cell-to-cell spread in epithelial and neuronal tissues (Johnson and Feenstra, 1987; Mettenleiter *et al.*, 1987b; Neidhardt *et al.*, 1987; Johnson *et al.*, 1988; Kudelova *et al.*, 1991; Card *et al.*, 1992; Jacobs *et al.*, 1993; Whealy *et al.*, 1993; Balan *et al.*, 1994; Dingwell *et al.*, 1994; Kritas *et al.*, 1994; Mulder *et al.*, 1994; Cohen and Nguyen, 1997; Tirabassi *et al.*, 1997; Dingwell and Johnson, 1998; Kimura *et al.*, 1998; Tirabassi and Enquist, 1998; Polcicova *et al.*, 2005). HSV and PRV gE/gI complexes are required for efficient spread of viruses between certain cultured epithelial cells, neurons, and other polarized cells with extensive cell junctions but are not needed for spread between highly transformed, nonpolarized cells, such as Vero or HeLa cells, which do not form cell junctions (Mettenleiter *et al.*, 1987a; Zsak *et al.*, 1992; Dingwell *et al.*, 1994; Dingwell *et al.*, 1995; Tirabassi *et al.*, 1997; Wisner *et al.*, 2000). For example, plaques formed by a gE-negative HSV mutant on monolayers of a keratinocyte cell line included eightfold fewer cells than plaques produced by wild-type HSV-1, yet there was no difference in cell-to-cell spread in monolayers of HeLa cells (Wisner et al., 2000). Moreover, PRV and HSV gE/gI complexes are required for spread within synaptically connected neuronal circuitry in the peripheral and central nervous systems (Whealy *et al.*, 1993; Mulder *et al.*, 1994; Dingwell *et al.*, 1995; Babic *et al.*, 1996; Tirabassi *et al.*, 1997; Tirabassi and Enquist, 1998). gE and gI are extensively complexed in virus-infected cells (Johnson and Feenstra, 1987; Johnson et al., 1988), and it is the gE/gI complex that functions in cell-to-cell spread (Johnson and Feenstra, 1987; Johnson *et al.*, 1988; Zuckermann *et al.*, 1988; Dingwell *et al.*, 1994; Dingwell *et al.*, 1995; Tirabassi *et al.*, 1997; Olson and Grose, 1998). In contrast to their effects on cell-to-cell spread, HSV and PRV gE/gI

complexes do not appear to be required for entry of cell-free virus, i.e., virus particles applied to the apical surfaces of cells (Mettenleiter *et al.*, 1987a; Dingwell *et al.*, 1994). HSV-1 gE/gI localizes specifically to the TGN during early phases of infection but moves out to cell junctions at intermediate to late times. One mechanism by which gE/gI facilitates cell-to-cell spread of HSV-1 involves the sorting of newly assembled virions to lateral cell surfaces and cell junctions. Mutant HSV-1 lacking gE accumulated more extensively in the cytoplasm, at apical cell surfaces, and in cell culture supernatants than did wild-type HSV-1 particles, which were found predominantly at cell junctions (Kimura *et al.*, 1998; Alconada *et al.*, 1999).

The gE/gI complex is also known as the HSV Fc Receptor (FcR) because of its high affinity for the Fc portion of IgG. Through binding of the Fc portion of IgG, the gE/gI complex contributes to the immune evasion capabilities of HSV-1, reducing the effectiveness of antibody-dependent cellular cytotoxicity (ADCC) targeting the virus and infected cells (Eberle et al., 1995).

Glycoprotein G

The 716 bp long US4 gene encodes the 238 aa glycoprotein G (gG). During infection, gG is incorporated into nuclear and cytoplasmic membranes in the cell (Frame *et al.*, 1986; Sullivan and Smith, 1987). The relevant function of gG in the herpesvirus life cycle has been difficult to identify. Mutant HSV-1 lacking US4 showed no discernible phenotypic abnormalities relative to the wild-type HSV-1 virus in non-polarized cells in culture, and the absence of gG resulted in only marginal attenuation of the virus in certain *in vivo* models (Atkinson *et al.*, 1978). However, at the primary site of infection, HSV-1 is required to infect the apical surfaces of mucosal epithelial cells. Recombinant viruses lacking gG were shown to be defective in the gC-dependent infection of the apical surfaces of polarized epithelial cells in culture and also in infection of the apical surfaces of corneal epithelial cells *in vivo* (Tran et al., 2000). It is not

known whether the gG function in infection is mediated in a cooperative manner with gC, whereby gG would assist with gC binding, or in an independent manner, whereby gG would be necessary in a second step that renders the gC interaction irreversible.

Glycoproteins H and L

The 2517 bp UL22 gene encodes the 838 aa glycoprotein H (gH). gH is a type I membrane glycoprotein containing an 18 aa signal peptide, a long 785 aa ectodomain, a single 21 aa transmembrane hydrophobic domain close to the C-terminus, and a 14 aa C-terminal cytoplasmic tail. The 675 bp UL1 gene encodes the 224 aa glycoprotein L. gL contains a 25 aa signal peptide; however, unlike other herpesvirus glycoproteins, gL does not contain a transmembrane domain. It appears that gL is not an integral membrane protein; rather, its membrane association and incorporation into virus particles is dependent on its interaction with gH (Dubin and Jiang, 1995). Infected cell membranes contain a heterodimer of gH and gL (gH/gL). When gH is expressed in transfected cells in the absence of gL, the resulting gH polypeptide is neither folded nor processed correctly. The malformed gH remains in the endoplasmic reticulum and undergoes self-aggregation (Foa-Tomasi et al., 1991; Forrester et al., 1991; Roberts et al., 1991). Similarly, cells infected with a gL-null virus do not produce virions containing gH. When cells are infected with a gH-null virus, gL is neither correctly processed nor incorporated into the plasma membrane or viral envelope (Hutchinson *et al.*, 1992a; Roop *et al.*, 1993). Due to the lack of a transmembrane region, the gL polypeptide is secreted into the medium (Dubin and Jiang, 1995). When both UL22 and UL1 are co-transfected into cells, the antigenic conformation of the gH/gL heterodimer is identical to that of virus infected cells, indicating that no other viral proteins are necessary for heterodimer formation. The mutual interaction of both glycoproteins is mediated by the N-terminal region of the first 69 aa of gL after cleavage of the 25 aa signal peptide (Roop *et al.*, 1993), while gH interacts through a

central region of its extra-cellular domain. C-terminal cysteine residues are required for proper gH/gL function; however the N-terminal cysteines, which are located within the putative gH/gL binding site are not required for gH/gL interaction or function (Cairns et al., 2005). A properly formed gH/gL heterodimer is required for both virus entry and virus-induced cell-to-cell fusion. Although viruses lacking gH/gL are unable to enter cells, they are able to attach to the cell surface. Therefore, the role of gH/gL in virus entry is during the virus envelope-plasma membrane fusion event and not required for virion attachment or receptor binding. An interesting result concerning the mechanism of gH/gL functions was obtained when using anti-gL mAbs directed to the C-terminal portion of gL. Certain mAbs inhibited virus-induced cell-to-cell fusion resulting from syncytial mutations but not virus entry, indicating a fundamentally different mechanism may be at work in the two processes (Novotny *et al.*, 1996).

Glycoprotein J

The 279 bp US5 gene encodes the 92 aa glycoprotein J (gJ) (Ghiasi et al., 1998). gJ contains a predicted signal peptide as well as a hydrophobic transmembrane domain. The current function of gJ is unknown. Deletion viruses that lack the US5 ORF show no phenotypic deficiencies either *in vitro* or *in vivo* (Balan et al., 1994). More specifically, a gJ-null HSV-1 was capable of multiplying from an inoculation site in mice and entering and replicating in the peripheral and central nervous system (Balan et al., 1994). However, it has been shown that another gene in the unique long (UL) region of the viral genome, UL27.5, has amino acid sequences common to US5. UL27.5 was discovered due to the fact that antibodies generated against the 23 kDa gJ cross reacted with an unknown 43 kDa product (Chang et al., 1998). In contrast to gJ, UL27.5 accumulates in the cytoplasm of infected cells; nevertheless, it may be possible that UL27.5 and gJ could function redundantly and an observable change in phenotype would not be seen unless both US5 and UL27.5 were disrupted.

Glycoprotein K

The 1017 bp UL53 gene encodes the 338 aa precursor of glycoprotein K (gK) (Debroy *et al.*, 1985; Pertel and Spear, 1996). gK contains a cleavable 30 aa signal sequence as well as two asparagine residues at positions 48 and 58 that are glycosylated by N-linked mannose (Hutchinson et al., 1992b; Ramaswamy and Holland, 1992). gK is extremely hydrophobic and was originally proposed to contain four transmembrane domains, with both the N-terminus and C-terminus predicted to lie on the lumenal/extra-cellular side of cellular membranes (Debroy *et al.*, 1985). In contrast, experiments with *in vitro* translated gK in the presence of microsomal membranes predicted gK to contain three transmembrane domains, with the C-terminal tail located intracellularly (Debroy *et al.*, 1985; Ramaswamy and Holland, 1992; Mo and Holland, 1997). However, recent experiments by our laboratory utilizing epitope tags inserted into specific regions of gK confirmed the original prediction for the membrane topology of gK (Foster *et al.*, 2003).

Original attempts to isolate gK-deficient viruses through substitution of UL53 with the *lacZ* gene were unsuccessful and it was determined that gK was essential for HSV replication *in vivo* (MacLean *et al.*, 1991b). A subsequent attempt at the isolation of a F-strain gK-null virus was successful, resulting in an insertion-deletion mutation in gK that expressed a fusion protein containing the N-terminal 112 aa of gK. The aberrant virions were significantly less infectious and did not reach the extra-cellular space, with a high number of naked capsids and aberrant virions in the cytoplasm (Hutchinson and Johnson, 1995). Furthermore, cells infected with the F strain gK-null virus caused cell fusion in 143TK- cells. However, a KOS strain containing a more precise deletion of the UL53 gene resulted in a slightly different phenotype (Jayachandra *et al.*, 1997). While the egress of virions in the KOS gK-null virus was still severely impaired relative to a wild-type virus, the KOS gK-null did not cause cell fusion in 143TK- cells. A KOS

virus that was constructed to mirror the F strain insertion-deletion resulted in syncytia formation in 143TK- cells, so the syncytial phenotype of the "gK-null" viruses was attributed to the expression of the N-terminal 112 aa of gK (Jayachandra *et al.*, 1997). Importantly, a precise insertion-deletion gK mutation recently constructed in an F-strain BAC was not syncytial and showed a similar egress defect, confirming previous results (Melancon et al., 2005).

Studies of gK localization in infected cells had previously shown that gK was exclusively localized in the peri-nuclear spaces and contained unprocessed carbohydrates added in the rough endoplasmic reticulum, indicating that gK was not transported to the golgi complex and never reached the cell surface (Hutchinson *et al.*, 1995). Interestingly, while spontaneous mutations leading to syncytia formation can arise in UL20 (Baines *et al.*, 1991; MacLean *et al.*, 1991b), UL24 (Sanders *et al.*, 1982; Jacobson *et al.*, 1989), UL27 (gB) (Bzik *et al.*, 1984; Pellett *et al.*, 1985a), and UL53 (gK) (Ruyechan *et al.*, 1979; Bond and Person, 1984; Pogue-Geile *et al.*, 1984; Debroy *et al.*, 1985), a large majority of syncytial mutations map to the UL53 (gK) gene. The number of syncytial mutation found in gK would seem to be at odds with data indicating that gK never reaches the surface of infected cells; however, data obtained in our laboratory utilizing viruses engineered to contain epitope tags in the putative extra-cellular domains of gK indicates that gK does indeed reach the surface of infected cells, resolving the paradox of how gK could potentially function in virus induced cell-to-cell fusion (Foster *et al.*, 2003). In transiently transfected cells, gK localized in the ER and did not reach the cell surface. It was originally reported for PrV that gK is not correctly processed in a UL20 deletion mutant, and that coexpression of gK and UL20 restored gK processing at least partially (Dietz et al., 2000). In support of these original findings, our laboratory showed that coexpression of gK and UL20 in transfected cells restored the transport of gK to cell surfaces (Foster *et al.*, 2003). Nevertheless, while coexpression of UL20 also restored the transport of syncytial gK genes in transfected cells,

syncytia formation did not result in these experiments, indicating that additional viral proteins are needed to activate the membrane fusion potential of gK (Foster *et al.*, 2003). Recent studies from our laboratory have shown that gK and UL20 physically interact with each other and that this interaction is essential for their intracellular transport, cell-surface expression and functions in cytoplasmic virion envelopment (Foster *et al.*, 2004b).

Glycoprotein M and Glycoprotein N

The 1422 bp UL10 gene encodes the 473 aa glycoprotein M (gM), which contains eight predicted hydrophobic transmembrane regions (Baines and Roizman, 1993; Babic *et al.*, 1996). gM is a component of the virion envelope and is present in the cytoplasmic membranes of infected cells (MacLean *et al.*, 1993). However, recent evidence suggests a role for gM in nuclear egress (Baines et al., 2007). Viruses deficient in the expression of gM exhibit a 10 to 20 fold reduction in virus titers in Vero or BHK cells and form slightly smaller plaques (MacLean *et al.*, 1991b; MacLean *et al.*, 1993). The 276 bp UL49.5 (UL49A) gene encodes a 91 aa integral membrane protein (Baines and Roizman, 1993). The UL49.5 polypeptide contains a 23 aa cleaved signal peptide and a C-terminal hydrophobic transmembrane domain (Barnett et al., 1992). There are also potential sites for *O*-glycosylation, leading to the naming of UL49.5 as glycoprotein N (gN) in some cases. In PrV, the UL49.5 homolog was found to be *O*-glycosylated and incorporated into virions as a 14kDa envelope protein (Jons et al., 1996). However, the UL49.5 gene product showed no evidence of glycosylation in HSV-1 infected cells, and appeared to be abundantly present in virions, linked to the tegument by disulfide bonds (Adams et al., 1998). While initial attempts at deletion of UL49.5 were unsuccessful and indicated the possibility that UL49.5 was required for virus replication (Barker and Roizman, 1992), a more recent attempt utilizing a more precise deletion resulted in a UL49.5-deficient

virus that showed no phenotypic differences in plaque yield or morphology and only a marginal two-fold difference in virus yield (Adams et al., 1998).

gM and UL49.5 (gN) are conserved throughout alpha, beta-, and gammaherpesviruses and have been shown to form a complex in a number of herpesviruses analyzed to date (Jons *et al.*, 1998; Lake *et al.*, 1998; Wu *et al.*, 1998; Mach *et al.*, 2000; Koyano *et al.*, 2003). The PrV gM has been shown to act as an inhibitor of cell fusion resulting from cotransfection of either the PrV gB, gD and gH/gL or the F protein of bovine respiratory syncytial virus, indicating a general mechanism of fusion inhibition by gM (Klupp *et al.*, 2000b). While no definitive interaction has been shown between the HSV-1 gM and UL49.5, an enhancement of function has been demonstrated when both are present, inasmuch as cotransfection of the HSV-1 gM and UL49.5 significantly inhibited cell fusion caused by the HSV-1 gB, gD and gH/gL or the Molony murine leukemia virus envelope protein, while transfection of the HSV-1 gM alone did not inhibit membrane fusion (Koyano et al., 2003). A requirement of both gM and gN for inhibition of cell fusion was also shown for HHV-8 (Koyano et al., 2003). At this point in time, the mechanism by which gM and UL49.5 mediate inhibition of cell-to-cell fusion is unknown.

Characterization of the UL20 Protein (UL20p)

The UL20 ORF: The UL20 open reading frame is situated between the UL19 gene and the recently confirmed UL20.5 gene in the unique long region of the HSV-1 genome. UL20 is expressed as a γ1 (late) gene that does not require viral DNA replication for expression (Ward et al., 1994), and is conserved in the alphaherpesviruses.

Membrane Topology of UL20p: Based on its predicted amino acid sequence, the UL20 protein was suggested to be an integral membrane protein containing two or three hydrophobic transmembrane domains (McGeoch et al., 1988). However, when the hydrophobic and membrane-spanning domains of UL20p were calculated by using the TMPred and SOSUI

computer algorithms (Hofmann and Stoffel, 1993; Hirokawa *et al.*, 1998) and used to derive a predicted membrane spanning model of UL20p, four hydrophobic regions were predicted, with no predicted signal peptide. This putative UL20p model features four membrane spanning regions, placing both the 66 amino acid amino terminus and the 14 amino acid carboxyl terminus of UL20p within the cytoplasm (domains I and V). In addition, a third small 10 amino acid domain is predicted to be located intracellularly (domain III), while the two other domains are predicted to be located extracellularly (domains II and IV). Domain II is predicted to contain 7 amino acids, while domain IV is predicted to contain 32 amino acids. As the largest extra-cellular domain, domain IV holds the best chance for interactions with the extra-cellular portions of other membrane proteins. This predicted model of the UL20p membrane topology has been partially confirmed in our laboratory with FLAG epitope tags located either on the N-terminus of UL20p, shown to be intracellular, or inside of domain IV, shown to be present on the extra-cellular/lumenal side of cellular membranes (Foster et al., 2004b).

UL20 Interdependence With gK for Cell Surface Expression and Internalization to the Trans-Golgi Network: Initial reports on the cellular localization of the UL20 protein indicated that it was not expressed at the cell surface but was present in virions purified from extra-cellular fluid and from the cytoplasm. The UL20 protein was found to be present in the nuclear membranes, in the Golgi apparatus, and dispersed in the cytoplasm, but was not detected in the plasma membranes of infected cells (Ward et al., 1994). In contrast, recent experiments performed by our laboratory show that independently expressed gK or UL20p failed to be transported from the ER in Vero cells. Similarly, infection of Vero cells with either a gK-null or UL20-null virus resulted in ER entrapment of UL20p or gK, respectively. In contrast, transiently coexpressed gK and UL20p predominantly localized to the TGN, and TGN-localized gK and UL20p were shown to originate from internalized gK and UL20p expressed at cell surfaces. In

all experiments, UL20p and gK were seen to colocalize, consistent with the hypothesis that gK and UL20p directly interact and that this interaction is important for their TGN localization and function in the virus life cycle (Foster et al., 2004b).

Function of UL20p in the HSV-1 Lifecycle: The original description of the role of UL20p in the virus life cycle was predicated on the observation that an F-strain UL20-null virus, R7225, had a peri-nuclear block in virus egress, resulting in a large accumulation of virions between the inner and outer nuclear lamellae as well as an abnormal level of un-enveloped capsids in the cytoplasm. In addition, the R7225 virus formed small syncytia on 143TK- cells (Baines et al., 1991). However, at the time it was unknown that the UL20.5 gene was located adjacent to UL20, and the R7225 virus contains an aberrantly fused gene consisting of the 5' half of the UL20.5 ORF and the 3' half of the UL20 gene. As such, it was unknown whether or not the description of the UL20-null phenotype could be attributed to the aberrant gene. To resolve the true phenotype of an HSV-1 virus lacking the UL20 gene, our laboratory constructed an insertion-deletion mutation into the HSV-1 KOS-strain UL20 gene that consisted of removal of the 5' region of the UL20 ORF, while maintaining the integrity of both the UL20.5 gene and the promoter for the major capsid protein, UL19. In accordance with the R7225 virus, the KOS strain UL20-null virus exhibited a defect in virion egress; however, the block in virion egress consisted solely of an accumulation of un-enveloped capsids in the cytoplasm, and no accumulation of peri-nuclear virions was observed (Foster et al., 2004a). Furthermore, the KOS UL20-null virus did not form syncytial plaques on 143TK- cells. The observable phenotypes of the KOS UL20-null virus were in agreement with the phenotype described for a PrV virus lacking UL20 (Fuchs et al., 1997), indicating that the previously described phenotype of the R7225 virus was in all likelihood due to the presence of the aberrantly fused gene. In addition, we found that the UL20 protein was required for virus-induced cell-to-cell fusion resulting from

syncytial mutations in either gB or gK, implying that UL20 may serve to regulate membrane fusion events in HSV-1 (Foster et al., 2004a).

Virus-Induced Syncytia Formation

While wild-type herpesviruses usually cause infected cells to round up and clump together, some viral mutants in both HSV-1 and HSV-2 cause cells to fuse into large polykaryocytes containing many nuclei (Hoggan and Roizman, 1959; Ejercito *et al.*, 1968; Ryechan *et al.*, 1979). Virus-induced cell-to-cell fusion has been extensively studied for several reasons: as a probe of the structure and function of the cellular membranes of cells during infection reflected in their behavior, as a tool for analysis of the functions of viral membrane proteins, and as a model of the initial interaction between HSV and susceptible cells that results in fusion of the viral envelope with the cellular plasma membrane (Roizman, 1962; Campadelli-Fiume and Serafini-Cessi, 1985). Herpes simplex viruses specify at least eleven virally encoded glycoproteins: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and potentially gN (UL49.5). HSV also encode several non-glycosylated membrane associated proteins: UL20, US9, UL24, UL43, and UL34. Herpesvirus membrane proteins function in several important roles including virus entry via fusion of the viral envelope with cellular membranes, intracellular virion morphogenesis and egress, cell-to-cell spread, and virus-induced cell fusion (Mettenleiter, 2002a; Mettenleiter, 2002b; Roizman and Knipe, 2001; Spear, 2004; Spear and Longnecker, 2003). At least 11 membrane proteins are present on the surface of the virion envelope (Roizman and Knipe, 2001).

Syncytia formation can be viewed as an aberrant manifestation of the interactions of altered membranes in herpesvirus infected cells with the unaltered membranes of neighboring cells. Genetic analysis has shown that mutations that cause extensive virus induced cell-to-cell fusion map to at least four and possible more loci within the viral genome: the UL20 gene

(Baines and Roizman, 1991; Melancon *et al.*, 2004), the UL24 gene (Sanders *et al.*, 1982; Jacobson *et al.*, 1989), the UL27 gene encoding glycoprotein B (gB) (Bzik *et al.*, 1984; Pellett *et al.*, 1985b), and the UL53 gene coding for glycoprotein K (gK) (Ryechan *et al.*, 1979; Bond and Person, 1984; Pogue-Geile *et al.*, 1984; Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987; Hutchinson *et al.*, 1995), with the great majority of syncytial mutations arising in either UL27 (gB) or UL53 (gK).

The mechanisms involved in virus-induced cell fusion are unclear, but some progess has been made on understanding the components required for cell fusion resulting from syncytial mutations in UL27 or UL53. In one set of experiments, the requirements for syncytia formation caused by a mutation at codon 855 of gB (gB^{ANG}) were analyzed. In Vero cells, gC, gG, gJ, and UL43 were shown to be dispensable for syncytium formation at both high and low multiplicities of infection, while gD, gH, gL, gE, gI, and gM were all required for the fusion of cellular membranes (Davis-Poynter *et al.*, 1994). These results confirmed that the requirements for virion entry and cell-to-cell fusion are not identical. While gD and gH/gL, like gB, are essential for both virion entry and virus-induced cell fusion, gE, gI, and gM are dispensable for virus penetration, yet can play a role in cell-to-cell spread (Davis-Poynter *et al.*, 1994). In addition, it has been shown that syncytial mutations in gK failed to cause virus-induced cell fusion in the absence of gB, indicating that gB was essential for gK-associated cell-to-cell fusion (Cai *et al.*, 1988a); Cai *et al.,* 1988). Recently, our laboratory demonstrated a requirement for UL20 in virus-induced cell fusion caused by syncytial mutations in either gB or gK. We also showed the role of gK in syncytia formation caused by gB syncytial mutations (Melancon *et al.*, 2005).

Breast Cancer

Cancer is an important factor in the global burden of disease, and breast cancer incidence has its wide share in the pie chart of cancer incidence. Breast cancer is the formation of a

malignant tumor that starts from cells of the breast. The disease occurs almost entirely in women, but men can get it, too.

Breast Cancer Statistics

Breast Cancer is the second leading cause of cancer deaths in women today (after lung cancer) and is the most common cancer among women, excluding nonmelanoma skin cancers. According to the World Health Organization, more than 1.2 million people will be diagnosed with breast cancer each year worldwide and over 500,000 will die from the disease. According to the current statistics of the Centers for Disease Control and Prevention, breast cancer accounts for 32 percent of all female cancers and is responsible for 18 percent of cancer deaths in women. The National Cancer Institute estimates that about 1 in 8 women in the United States (approximately 13.3 percent) will develop breast cancer during her lifetime. This estimate is based on cancer rates from 1975 through 2004 (NCI, 2007). The American Cancer Society estimates that in 2007 approximately 178,480 new invasive cases of breast cancer will be diagnosed among women in the United States (2,820 of those cases will be diagnosed in Louisiana). An estimated 40,460 women will die from breast cancer (730 women will die in Louisiana) (ACS, 2007).

Breast Cancer Types

Each breast has 15 to 20 sections called lobes, which have many smaller sections called lobules. The lobes and lobules are connected by thin tubes, called ducts (Figure1.8). The most common type of breast cancer is ductal cancer. It is found in the cells of the ducts. Cancer that starts in lobes or lobules is called lobular cancer. It is more often found in both breasts than other types of breast cancer. Cancers also are classified as non-invasive (in situ) and invasive (infiltrating). The term *in situ* refers to cancer that has not spread past the area where it initially developed. Invasive breast cancer has a tendency to spread (metastasize) to other tissues of the

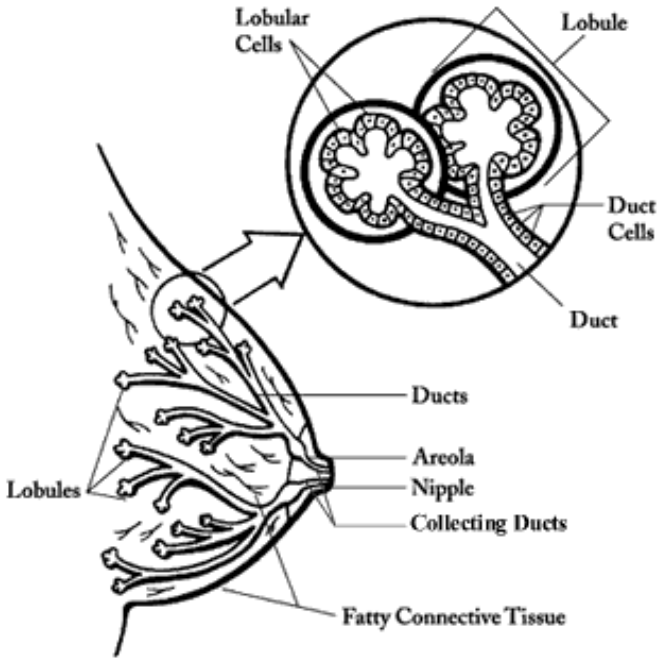

Figure 1.8: The structure of the female breast.

breast and/or other regions of the body. A less common type of breast cancer is inflammatory breast cancer characterized by general inflammation of the breast. Other rare types of breast cancer are medullary carcinoma (an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue), mucinous carcinoma (formed by the mucus-producing cancer cells), tubular carcinoma, etc. (WHO, 1981).

Historical Overview

While the incidence of breast cancer as well as the recovery rate continues to rise, breast cancer is hardly a new affliction (Harvey, 1974; Kardinal and Yarbro, 1979; Gallucci, 1985). The recorded history of breast cancer traces back thousands of years. It is no surprise that from the dawn of history doctors have written about cancer. Incidents of breast cancer have been documented back to the early Egyptians when the popular treatment was cautery of the diseased

tissue. Surgery was practiced but it was an extremely radical treatment considering there was no anesthesia or antisepsis available.

The oldest description of cancer (although the term cancer was not used) was discovered in Egypt and dates back to approximately 1600 B.C. The Edwin Smith Papyrus describes 8 cases of tumors or ulcers of the breast that were treated by cauterization, with a tool called “the fire drill”. The writing says about the disease: “There is no treatment”.

The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 B.C.), the "Father of Medicine". Hippocrates used the terms carcinos and carcinoma to describe non-ulcer forming and ulcer-forming tumors. In Greek these words refer to a crab, most likely applied to the disease because the finger-like spreading projections from a cancer called to mind the shape of a crab. Carcinoma is the most common type of cancer.

According to the doctrines of the Greek physician Caudius Galen (130-200 AD), whose works on physiology and anatomy dominated medical thought until the Middle Ages, melancholia was the chief factor in the development of breast cancer. Special diets were the recommended treatment. However, other treatments included exorcism and the use of topical applications which were seldom preferred by patients.

During the Renaissance, Andreas Vesalius recommended mastectomy as well as ligatures (sutures) to control the bleeding rather than cautery. Recognition that breast cancer could and did spread to the regional auxiliary nodes was first recognized by the physician LeDran (1685-1770). Dr. LeDran was likely the first to associate poor prognosis with the spread of breast cancer to the lymph nodes.

The famous Scottish surgeon John Hunter (1728-1793) suggested that some cancers might be cured by surgery and described how the surgeon might decide which cancers to operate

on. If the tumor had not invaded nearby tissue and was “moveable”, he said, “There is no impropriety in removing it”.

During the mid 1800’s, surgeons first began to keep detailed records of breast cancer. Those statistics indicate that even those treated by mastectomy had a high rate of recurrence within eight years—especially when the glands or lymph nodes were affected. Nevertheless, the common treatment was to remove the breast and the surrounding glands in an effort to stave off any further tumor development.

In 1894 William Roentgen discovered X-rays. This paramount discovery shed light on the detection of many diseases as well as breast cancer. Some years later, in 1913, Albert Solomon, a pathologist in Berlin, produced images of 3,000 gross mastectomy specimens. He observed black spots at the centers of breast carcinomas (microcalcifications).

Between the 1930’s and the 1950’s treatment and detection improvements were noticeable. This was the time when Stafford Warren (Rochester memorial Hospital, New York) developed a stereoscopic system for tumor identification. Also, doctors started classifying the stage and progression of breast cancer. In 1949 Raul Leborgne (Uruguay) emphasized breast compression for identification of calcifications. In 1940s-1950s breast self-examinations were advocated.

In 1960 Dr. Robert Egan (Houston) adapted high-resolution industrial film for mammography, allowing simple and reproducible mammograms with improved image detail. And in 1963 the first randomized controlled trial of screening by the Health Insurance Plan of New York found that mammography reduced the 5-year breast cancer mortality rate by 30 percent. Major improvements in mammography equipment, such as reduced radiation dosage, digital imaging, and computer-aided diagnosis, improved detection of breast cancer.

Breast Cancer Risk Factors

Every woman is at risk for developing breast cancer. Several relatively strong risk factors for breast cancer that affect large proportions of the general population have been known for some time. However, the vast majority of breast cancer cases occur in women who have no identifiable risk factors other than their gender (Kelsey and Gammon, 1990).

The "established" risk factors for breast cancer are female gender, age, previous breast cancer, benign breast disease, hereditary factors (family history of breast cancer), early age at menarche, late age at menopause, late age at first full-term pregnancy, postmenopausal obesity, low physical activity, race/ethnicity and high-dose exposure to ionizing radiation early in life.

The "speculated" risk factors for breast cancer include never having been pregnant, having only one pregnancy rather than many, not breast feeding after pregnancy, use of postmenopausal estrogen replacement therapy or postmenopausal hormone (estrogen/progestin) replacement therapy, use of oral contraceptives, certain specific dietary practices (high intake of fat and low intakes of fiber, fruits, and vegetables, low intake of phytoestrogens), alcohol consumption, tobacco smoking, and abortion.

Although men can and do develop breast cancer, the disease is 100 times more likely to occur in a woman than in a man. Women are at a higher risk of breast cancer because they have much more breast tissue than men do. Also, estrogen promotes the development of breast cancer.

Stages of Breast Cancer

The staging systems currently in use for breast cancer are based on the clinical size and extent of invasion of the primary tumor (T), the clinical absence or presence of palpable axillary lymph nodes and evidence of their local invasion (N), together with the clinical and imaging evidence of distant metastases (M). This is then translated into the TNM classification which has been subdivided into Stage 0 called carcinoma in situ (lobular carcinoma in situ (LCIS) and

ductal carcinoma in situ (DCIS) and four broad categories by the Union Internationale Centre Cancer (UICC), which are the following:

Stage I – early stage breast cancer where the tumor is less than 2 cm across and hasn't spread beyond the breast.

Stage II – early stage breast cancer where the tumor is either less than 2 cm across and has spread to the lymph nodes under the arm; or the tumor is between 2 and 5 cm (with or without spread to the lymph nodes under the arm); or the tumor is greater than 5 cm and hasn't spread outside the breast.

Stage III – locally advanced breast cancer where the tumor is greater than 5 cm across and has spread to the lymph nodes under the arm; or the cancer is extensive in the underarm lymph nodes; or the cancer has spread to lymph nodes near the breastbone or to other tissues near the breast.

Stage IV – metastatic breast cancer where the cancer has spread outside the breast to other organs in the body.

The Past and the Future of Breast Cancer Detection

Increased breast cancer awareness with breast self-examinations and major improvements in routine breast cancer screening had a paramount effect on early detection of breast cancer. Improvements in conventional mammography (an x-ray technique to visualize the internal structure of the breast) such as the low radiation dosage, enhanced image quality, development of statistical techniques for computer-assisted interpretation of images, long-distance electronic image transmission technologies (telemammography/teleradiology) for clinical consultations, and improved image-guided techniques to assist with breast biopsies (the removal of cells or tissues for examination under a microscope) continue to lower the morbidity and mortality of breast cancer. The support of research on technologies that do not use x-rays and are not used for

routine breast cancer screening, such as magnetic resonance imaging (MRI), ultrasound, and breast-specific positron emission tomography (PET) may play a considerable role in further improvements of breast cancer early detection. In most cases, the earlier breast cancer is detected, the better the survival rate. Today mammography is the best available method to detect breast cancer in its earliest, most treatable stage - an average of 1.7 years before the woman can feel the lump. Generally, treatment is most effective before the disease spreads. When breast cancer is diagnosed at a local stage, the 5-year survival rate is greater than 90%. This rate decreases to less than 50% when the disease has spread to the lymph nodes and less than 20% when it has spread to distant organ sites.

Despite recent progress in early detection and surgical therapy, the mortality due to breast cancer has changed little over the past decades, primarily because the occult dissemination of cancer cells can occur at an early stage of carcinogenesis. Occult dissemination of tumor cells in patients with operable cancer can subsequently lead to formation of metastasis, yet it is usually missed by conventional tumor staging. The success of routine mammography screening for breast cancer is that it involves increasingly more patients with small primary tumors formerly thought to have an overall excellent prognosis. Yet, only approximately two thirds of these patients actually have this favorable prognosis, while the remaining third develops metastatic disease. Thus, there is emerging evidence that tumor cells can disseminate into secondary organs at an earlier stage of primary tumor development than appreciated by current risk classifications. There are several challenges that must be addressed in an effort to continue to lower the mortality associated with this disease.

Oncolytic Virotherapy

Cancer remains a serious threat to human health, causing over 500 000 deaths each year in US alone, exceeded only by heart diseases. Advances in molecular biology have defined

entirely new approaches to cancer therapy, utilizing molecular interventions. Neoplasms not responsive to current oncological treatment have become prime candidates for molecular-based therapies. One of the newest technologies being developed to fight cancer is the oncolytic virotherapy.

Combating cancers with viruses is a revolutionary approach undergoing testing in various pre-clinical and clinical models. Oncolytic viruses are restricted to replicate only in tumor tissue either by design (adenovirus and HSV-derived) or by the natural propensity of some strains (myxoma virus, reovirus, Newcastle disease virus, etc.) (Chiocca, 2002; Fulci and Chiocca, 2003). They can lead to tumor cell lysis with minimal infection replication potential in adjoining non-neoplastic tissue. Because of paramount safety concerns, first-generation oncolytic viruses were designed to be significantly attenuated in their lytic potential. Results from recent clinical trials have revealed the safety of this approach, but have underscored the urgency for design and testing of more tumor-selective and –potent viruses to realize the full therapeutic potential of this revolutionary treatment modality.

The earliest case reports of viral oncolysis document spontaneous regression of cervical cancer following rabies vaccination, published 100 years ago (Dock, 1904). In 1912, DePace described his work on uterine cervical carcinoma. He reported tumor regression after inoculation of an attenuated rabies vaccine (DePace, 1912). This historical report led to an animal experiment on tumor burden by Levaditi and Nicolau in 1920. They first documented vaccinia viral oncolysis in the mouse (Levaditi and Nicolau, 1922). Tumor lysis by Newcastle disease virus and influenza virus was observed in the 1940s. A human trial was performed by Pack in 1940 in New York using an attenuated rabies virus against melanoma that yielded a remarkable partial remission (Pack, 1950). By mid- 20th century, Southam in New York was treating large numbers of patients suffering from various types of malignancies, using numerous viruses

including myxovirus, paramyxovirus, arbovirus, etc. The cause of tumor regression induced by these viruses has been carefully documented by him (Southam and Moore, 1952; Newman and Southam, 1954; Southam and Moore, 1954; Southam *et al.*, 1958) . In one of his trials, oncolytic viruses were used to treat cancers in humans in a study involving injection of wild-type adenoviruses of different serotypes into patients with cervical cancer (Newman and Southam, 1954). Although tumor regression without toxicity was observed in more than half of the patients, disease progression was observed in all patients. Furthermore, later Southam reported toxicity during his trials of oncolytic virotherapy (Southam *et al.*, 1956). The absence of durable anti-tumor activity of viruses was mirrored in other human trials of that day (Cassel and Garrett, 1965) leading investigators to abandon this mode of therapy.

Virology was then at its infancy. In the years following these reports, advanced molecular biological techniques were developed backed by technical developments, allowing the assurance for safety of this type of therapy and anticancer effect by increased selectivity and inclusion of therapeutic genes. Oncolytic virotherapy subsequently gathered momentum in recent decades with advances in tumor biology, molecular biology, and molecular virology, which set the stage for the modern era of oncolytic viral therapy. Since then, multiple genetically engineered viruses have been evaluated for their potential as therapeutic agents in the treatment of malignant tumors (Andreansky *et al.*, 1997; Martuza, 2000; Fernandez *et al.*, 2002; Ring, 2002). Some of the viruses tested include adenovirus, HSV, vaccinia virus, reovirus, New-castle disease virus, vesicular stomatitis virus, measles virus, poliovirus and West Niles virus (Chiocca, 2002; Nemunaitis, 2002; Nemunaitis and Edelman, 2002; Kirn, 2003).

The effect of HSV-1 against a brain tumor model was demonstrated by Martuza in 1991 (Martuza *et al.*, 1991). Numerous viruses then began to be studied for their efficacy in tumor oncolysis in humans. Only a few years following the report by Martuza, the recombinant

adenovirus Onyx-15 underwent its first clinical trial in cancer patients. Multiple different types of oncolytic virus therapies followed this first adenoviral clinical trial.

Many gene therapy methods are being reported for their efficacy against cancer currently, but the oncolytic effect of a replication-competent virus stands out among such approaches, even without the inclusion of therapeutic genes. As a methodology for gene therapy, virus is a very important vector for carrying therapeutic genes. Oncolytic virus also has this capacity as a promoter gene element (e.g. CEA, Muc-1, Albumin) (Goldstein and Weller, 1988; Miyatake *et al.*, 1999; Mullen *et al.*, 2002; Kasuya *et al.*, 2004), enzyme-prodrug combination (e.g. cytosine deaminase, cytochrome P450) (Ichikawa *et al.*, 2001; Nakamura *et al.*, 2001), or immune-stimulating factor (e.g. IL12, GM-CSF) (Liu *et al.*, 2003; Wong *et al.*, 2004; Hu *et al.*, 2006). Including these therapeutic genes, oncolytic viruses are expected to produce major therapeutic effects for dealing with various cancers. Recent unlimited technological advances will pave a way for the development of further therapeutic transgenes as well as more potent and safer viruses for oncolytic virotherapy. Currently, the greatest obstacle to success is the insufficient therapeutic efficacy against cancer due to poor virus replication after injection into solid tumors. Numerous clinical trials using oncolytic viruses have already shown their safety as therapeutics. Encouraging results of many different phase I or II clinical trials have been reported from Western and Asian countries (Table 1.2). There are numerous ongoing phase III trials pending reports. The emphasis is on the efficacy in the ongoing studies nowadays.

HSV-1 as a Vector for Cancer Treatment

Broadly speaking, there are two types of HSV-1 vectors, and both have been used in cancer treatment. (1) Replication-defective vectors, in which transgene expression cassettes are inserted in a viral genome with one or a few essential viral genes deleted. Such gene therapy vectors can effectively express transgene products, but are unable to replicate, except in cells that

Table 1.2: Oncolytic Virus Clinical Trials

Virus	Target	Injection	Phase	Country	Reference
HF10	Solid	Direct	I	Japan	Nakao *et al.*, 2004
ONYX-015	Pancreatic C	Direct	I	USA	Mulvihill *et al.*, 2001
ONYX-015	Pancreatic C	Direct	I/II	USA	Hecht *et al.*, 2003
ONYX-015	Colorectal C	Venous	II	USA	Hamid *et al.*, 2003
ONYX-015	Liver Cancer	Venous	II	USA	Makower *et al.*, 2003
ONYX-015	Head & Neck C	Direct	II	USA	Khuri *et al.*, 2000
ONYX-015	Head & Neck C	Direct	I	UK	Ganly *et al.*, 2000
ONYX-015	Head & Neck C	Direct	II	USA	Nemunaitis *et al.*, 2000
ONYX-015	Head & Neck C	Direct	II	USA	Nemunaitis *et al.*, 2001
ONYX-015	Ovarian	Venous	I	UK	Vasey *et al.*, 2002
G207	Brain Tumor	Direct	I	USA	Markert *et al.*, 2000
OncoVexGM-CSF	Breast, HNC, Mela	Direct	I	UK	Hu *et al.*, 2006
1716	Brain Tumor	Direct	I	UK	Rampling *et al.*, 2000
1716	Brain Tumor	Direct	I	UK	Harrow *et al.*, 2004
1716	Brain Tumor	Direct	II	UK	Papanastassiou *et al.*, 2002
ONYX-015	Brain Tumor	Direct	I	USA	Chiocca *et al.*, 2004
PV701	Solid	Venous	I	USA	Pecora *et al.*, 2002
PV701	Solid	Venous	I	Canada	Lorence *et al.*, 2003
Reolysin	Solid	Direct	I	Canada	Carlson *et al.*, 2005
H101	Solid	Direct	I	China	Yuan *et al.*, 2003
H101	Solid	Direct	II	China	Xu *et al.*, 2003
NV1020	Colon C	Portal	I	USA	Kemeny *et al.*, 2006

complement the deleted viral functions *in trans*. A special group of replication-defective HSV vectors are amplicons (Federoff *et al.*, 1997; Fraefel *et al.*, 2000; Link *et al.*, 2000). In this approach, an expression cassette for the therapeutic transgene(s) is placed in a plasmid ('amplicon' plasmid) that contains the viral packaging/cleavage signals and HSV-1 origin of replication. Defective HSV-like particles that contain concatemerized plasmid DNA are produced in eukaryotic cells that supply viral functions *in trans*. (2) Conditionally replicating vectors, in which deletion of some nonessential viral genes results in viruses that preferentially infect, replicate in, and lyse tumor cells. Conditionally replicating vectors can also be modified to carry therapeutic transgenes to augment antitumor effects.

As mentioned above, oncolytic effect of a replication-competent virus stands out among various gene therapy approaches, even without the inclusion of therapeutic genes.

As mentioned earlier, the concept of viral lysis of tumor cells has been discussed for more than a century. Viral oncolysis is based on a simple yet elegant approach to cancer treatment. A principle consequence of the replicative cycle of selected viruses is cell lysis with subsequent spread to and lysis of adjacent tumor cells. Infection of a single cancer cell within a tumor, in theory, initiates a self-sustaining chain reaction resulting in infection and cell death throughout the tumor. The key to such an approach is genetically engineering viral attenuation such that the virus is predisposed to replicate cancer cells but not the adjacent normal tissue. In addition to its direct tumoricidal effect, oncolytic virus-mediated tumor destruction may provide an *in situ* source of tumor antigens for stimulating nonspecific and specific antitumor immunity which also triggers tumor destruction. Ideally, this mechanism should also inhibit the growth of tumor cells in metastatic loci in distant sites.

Advantages of HSV-1 as an Oncolytic Agent

HSV-1 has a number of intrinsic properties making it highly attractive for development as an oncolytic therapy for cancer. These include: (1) HSV-1 can infect and efficiently replicate in most actively dividing human cells such that oncolytic treatment with an appropriately engineered oncolytic version of HSV could allow treatment of most solid tumor types. (2) HSV-1 provides very robust lytic replication in permissive cells with a short replication time and as such provides a highly potent starting point for development as an oncolytic agent. In permissive cells, the entire replication cycle of HSV-1 is usually completed within 20h, releasing thousands of progeny virions upon cell lysis. (3) The large genome (~152 kb) is well characterized and contains many non-essential genes that can be mutated or replaced with large-sized individual or multiple therapeutic transgenes. (4) HSV-1 virions are capable of direct cell-to-cell spreading

through cell junctions in addition to spreading through the extracellular space. This feature is extremely useful for an oncolytic virus, as it allows for efficient viral penetration within solid tumors with minimal systemic spread. (5) The virus is very well characterized and effects in humans have been extensively studied. Therefore, its use as an oncolytic agent in humans should not provide surprises in the clinic. HSV-1 very rarely produces life-threatening medical illness in immune-competent adults. (6) Known mutations can supply tumor-selective replicative properties to the virus, rendering the virus nonpathogenic. (7) The vector can be administered repeatedly in immunocompetent hosts. (8) Viral DNA remains episomal and does not get integrated into the cellular genome. (9) The viral replicaation can be terminated by anti-herpes drugs, such as acyclovir, probably the most effective antiviral drug currently available. (10) There are mice and non-human primates that are susceptible to HSV-1 and can be used for preclinical evaluation for safety and efficacy.

Strategies for Creating Oncolytic HSV-1 Vectors

A number of strategies have been used to create tumor-selective oncolytic viruses (Kirn *et al.*, 2001; Nemunaitis and Edelman, 2002). The main strategies can be grouped into three general approaches. (1) To delete gene functions that are critical for efficient viral replication in normal cells but are dispensable in tumor cells. Many replication-conditional HSV-1 vectors have included mutations and/or deletions in one or more of the genes encoding thymidine kinase, DNA polymerase, uracil DNA glycosylase, ribonucleotide reductase (RR), and ICP 34.5 ($\gamma_1$34.5) (Lou, 2003). (2) To limit the expression of a critical viral gene to tumor tissues through the use of tumor- and/or tissue-specific promoters, promoter/enhancer sequences from genes that are overexpressed in cancers (Mullen *et al.*, 2002; Kasuya *et al.*, 2004; Reinblatt *et al.*, 2004). (3) To alter viral tropism through modification of surface proteins (Zhou *et al.*, 2002; Argnani *et al.*, 2004; Goins *et al.*, 2004).

While proof has been demonstarted in principle for the use of genetically engineered replication-competent HSV-1 for tumor oncolysis, startegies have evolved to futher improve the therapeutic ratio of these viruses by enhancing their efficacy for oncolytic virotherapy. One of the advantages of HSV-1 is the capacity to incorporate large and/or multiple transgenes within the viral genome. One of the strategies to improve efficacy of oncolytic HSV-1 involves the 'arming' of such viruses by the insertion of therapeutic genes into the virus genome. Oncolytic HSV-1 vectors have been used successfully to deliver immunoregulatory molecules, prodrug converting enzymes and angiogenesis inhibitors (Andreansky *et al.*, 1998; Parker *et al.*, 2000; Nakamura *et al.*, 2001; Wong *et al.*, 2001a; Liu *et al.*, 2003; Mullen *et al.*, 2004).

The other strategy involves combining viral therapy with standard cancer therapies (i.e. chemotherapy, radiation therapy). Combining therapies may have an additive effect , lowering dose requirements of either therapy and minimizing potential treatment-associated toxicity or tumor resistance to therapy (Blank *et al.*, 2002; Chung *et al.*, 2002; Cinatl *et al.*, 2003; Bennett *et al.*, 2004; Post *et al.*, 2004).

A large body of information on the functions of viral proteins in lytic infections owes its existence to the development of the first technique for site-directed HSV-1 gene deletions or insertions (Post and Roizman, 1981; Roizman and Jenkins, 1985). However, conventional homologous recombination techniques require time-consuming processes to create oncolytic HSV-1. The construction of HSV recombinants using a newly developed method, the bacterial artificial chromosome (BAC) system, promises the rapid generation of multiple HSV-1 vectors with desired mutations/deletions/transgene insertions yielding recombinant virus free of parental or wild-type virus (Horsburgh *et al.*, 1999; Tanaka *et al.*, 2003; Tischer *et al.*, 2006). This advance has revolutionized work with DNA viruses.

Most of the oncolytic HSV-1 vectors reported so far carry inactivating mutations in or deletion of ICP6 gene (UL39) and/or the ICP34.5 gene ($\gamma_1$34.5) (Markert *et al.*, 1993; Mineta *et al.*, 1994; Mineta *et al.*, 1995).

ICP6 is the large subunit of viral ribonuclotide reductase, an enzyme required for the synthesis of deoxyribonucleotide triphosphates (dNTPs) by the conversion of rNTPs to dNTPs which are necessary for the synthesis of new viral DNA during virus replication. This enzyme is overexpressed in most cancer cells. As a consequence, HSV-1 mutant lacking the ICP6 preferentially replicate within tumor cells that supply this missing function *in trans*.

ICP34.5, the HSV-1 neurovirulence factor, has been found to be essential for pathogenicity of HSV (Chou *et al.*, 1990; Chou and Roizman, 1992, 1994). The discovery of this gene has provided a considerable impetus for the use of HSV-1 for cancer therapy. Deletion of ICP34.5 provides the greatest degree of attenuation of any individual mutation where the virus can still replicate in actively dividing cells. Deletion of $\gamma_1$34.5 gene, present in two copies in the long repeat regions, reduced neurovirulence by greater than five orders of magnitudes upon direct intracranial inoculation in mice (Chou *et al.*, 1990). In response to viral infections, cells activate RNA-dependent protein kinase (PKR), which phosphorylates the alpha subunit of translation initiation factor 1 (eIF-2α). The latter blocks the translation of mRNA, and thus provides an innate host defense mechanism against viral infection. HSV-1 has developed an elegant mechanism to deal with PKR activation. The product of $\gamma_1$34.5 gene binds to protein phosphatase-1, resulting of dephosphorylation of eIF-2α, and precluding host protein synthesis shut-off (He *et al.*, 1997). An important fact is that the portion of the latency-associated transcripts (LAT) is encoded on the DNA strand opposite the $\gamma_1$34.5 genes. Thus, not only is the function of the $\gamma_1$34.5 gene compromised in $\gamma_1$34.5$^-$ HSV-1 mutants, but their capacity to express genes necessary for the virus to establish latency is lost. Survival of $\gamma_1$34.5$^-$ attenuated mutants is

restricted to replicating cancer cells which also lack intact apoptotic mechanisms (Chou *et al.*, 1990; Chou and Roizman, 1992). A variety of genetically engineered HSV constructs either deleted in both copies of $\gamma_1$34.5 or with mutations in this gene have been extensively evaluated both in vitro and in vivo for the treatment of tumors. However, viruses deleted for $\gamma_1$34.5 replicate sluggishly and do not replicate to very high titers in experimental tumor models. Expeirments with animal models as well as phase I/II clinical trials have revealed that viruses that have both copies of $\gamma_1$34.5 gene deleted do not spread efficiently within tumors, in comparison with their parental wild-type strains (Kramm *et al.*, 1997). This potential drawback has necessitated the need for construction of new improved HSV-1 oncolytic viruses. A current such strategy for the construction of improved oncolytic HSV-1 based on previously studied viruses includes the deletion of only one of the two $\gamma_1$34.5 genes, allowing for improved viral replication while the virus remains largely attenuated. Another drawback that should be addressed and taken into consideration while constructing novel oncolytic viruses is the fact that the antitumor activity of oncolytic HSV does not induce a significant bystander effect (the killing of uninfected cells after death of the infected neighboring cells). The antitumor activity of an oncolytic HSV-1 could be significantly enhanced if the virus incorporates a cell-membrane fusion function (Fu and Zhang, 2002; Nakamori *et al.*, 2004). Fusogenic viruses will produce syncytia formation in the tumor, therby directly enhancing the destructive power of the virus and promoting its intratumor spread (Bateman *et al.*, 2000; Higuchi *et al.*, 2000). Furthermore, in theory, this unique mechanism of tumor destruction in vivo by the fusogenic oncolytic HSV can induce strong antitumor immune responses, which can further facilitate tumor eradication (Nakamori *et al.*, 2004). Novel approaches for the construction of improved oncolytic HSV-1 with enhanced ability of the virus to spread to uninfected cells include the incorporation of virus-induced cell fusion abilities.

HSV-1 Oncolytic Viruses

Most of the viruses used in early studies to treat cancer were naturally attenuated or vaccine strains. The exact mechanisms of tumor-selectivity of these viruses were mainly unknown. It was only in 1991 that the first HSV-1 vector, dlsptk, genetically engineered for oncolytic virotherapy with clear rationale for tumor-preferring viral replication was reported (Martuza *et al.*, 1991). The study used a thymidine kinase (TK) deletion mutant strain of HSV-1. TK is involved in optimizing nucleic acid methabolism for virus growth. Wild-type HSV-1 carries its own TK gene which enables efficient replication of viral DNA in neuronal cells. Cellular TK is upregulated in actively dividing cells. Therefore, the HSV-1 viruses carrying deletions/mutations of TK gene selectively replicate in actively dividing tumor cells. Although the results of studies utilizing TK-mutated/deleted viruses, such as dlsptk, were promising (Martuza *et al.*, 1991; Markert *et al.*, 1992; Boviatsis *et al.*, 1994), TK mutants showed residual neurovirulence causing fatal encephalitis of the experimental animals not providing sufficient level of safety to be tested in human clinical trials. In tumor models in severe combined immunodeficient (SCID) mice, these mutants were shown to spread into normal tissues (Valyi-Nagy *et al.*, 1994b), indicating a lack of tumor specificity. In addition, they are resistant to commonly used anti-herpetic agents acyclovir and gancyclovir. The ongoing development of alternative mutant viruses over the last 17 years has been very active.

Many of the oncolytic HSV-1 vectors reported so far and tested as replicative anticancer agents carry inactivating mutations of the ICP6 (infected cell protein 6) gene UL39 encoding the large subunit of ribonucleotide reductase (Boviatsis *et al.*, 1994; Mineta *et al.*, 1994; Carroll *et al.*, 1996). This gene is also required for neurovirulence and is envolved in the conversion of rNTPs to dNTPs necessary for the synthesis of new viral DNA during replication, as mentioned earlier. The UL39-deleted mutant, hrR3, is significantly attenuated for replication in normal

cells, but replicates efficiently in cancer cells (Mineta *et al.*, 1994; Carroll *et al.*, 1996; Yoon *et al.*, 1998). This virus has shown efficacy in animal models of different cancers (Mineta *et al.*, 1994; Kasuya *et al.*, 1999; Yoon *et al.*, 2000; Jacobs *et al.*, 2001). Since ICP6 mutants are sensitive to acyclovir/gancyclovir, they have been tested in combination with gancyclovir (Mineta *et al.*, 1994; Kramm *et al.*, 1996; Carroll *et al.*, 1997). ICP6 mutants can cause fatal encephalitis if used at sufficient dose. Thus, like TK mutants, ICP6 mutants were not considered for human trials.

As mentioned, $\gamma_1$34.5 gene product (ICP34.5) was found to be essential for HSV-1 pathogenicity, deletion of which provides the greatest degree of attenuation of any individual mutation where the virus can still replicate in actively dividing cells, providing improved safety profile. ICP34.5 mutated viruses have been studied extensively by number of authors (Markert *et al.*, 1993; Chambers *et al.*, 1995; Kesari *et al.*, 1995; Randazzo *et al.*, 1995; Andreansky *et al.*, 1997).

HSV-1 1716 is one of the $\gamma_1$34.5-deleted mutants derived from strain 17+ and containing 759 bp deletions in both copies of the $\gamma_1$34.5 gene. It was found to be nonneurovirulent in mice (MacLean *et al.*, 1991a). 1716 has been tested in a number of tumor animal models showing therapeutic effects (Kesari *et al.*, 1995; Randazzo *et al.*, 1995; Lasner *et al.*, 1996; Kucharczuk *et al.*, 1997). The promising preclinical data were soon followed by a phase I clinical trial in glioma patients where the virus was injected intatumorally and was shown not to have adverse effects and improved the survival (Rampling *et al.*, 2000). In a further trial where patients were injected with 1716 some days prior to removal of injected tumor, virus replication was demonstrated in two of the tumors (Papanastassiou *et al.*, 2002). 1716 has been also tested in another clinical trial where it was injected intratumorally to melanoma patients (MacKie *et al.*, 2001).

R3616 has a strain F backbone and a 1-kb deletion in both copies of the $\gamma_1$34.5 gene (Chou *et al.*, 1990). This virus replicated efficiently in xenograft tumors, induced tumor regression and significantly prolonged animal survival (Chambers *et al.*, 1995; Andreansky *et al.*, 1996).

Considerable data produced by the use of the first generation viruses demonstrated promise for cancer therapy. The focus was on the treatment of intracranial glioma mainly, and there was a strong indication for clinical use. It was decided to construct the second generation viruses with the greatest possible level of safety for the use in humans. New mutant viruses with further deletions or alterations made to existing mutant strains were generated. The safety margin of vectors was increased and the likelihood of reversion to wild type was decreased. TK was left intact to retain acyclovir sensitivity. One of these viruses termed G207 (MediGene, Inc)(Mineta *et al.*, 1995) was entered into clinical trials for glioma therapy (Markert *et al.*, 2000). The promising safety data obtained from preclinical studies were concluded to warrant further clinical evaluation of G207 for the treatment of malignant gliomas in humans.

G207 virus is a version of HSV-1 strain F, deleted for both copies of $\gamma_1$34.5, and has an insertion of a *LacZ* gene into UL39 (ICP6). It should be noted that a nearly identical mutant, MGH-1, was constructed and tested separately by another group (Kramm *et al.*, 1997). G207 has been one of the most famous herpesviral vectors tested widely in both preclinical studies and clinical trials (Mineta *et al.*, 1995; Hunter *et al.*, 1999; Markert *et al.*, 2000; Todo *et al.*, 2000; Varghese *et al.*, 2001). G207 has several favorable properties for treatment of human tumors: replication competence in tumor cells, attenuated neurovirulence, temperature sensitivity, gancyclovir hypersensitivity, and the presence of the easily detectable histochemical marker (Mineta *et al.*, 1995).

Another multiple gene-deleted mutant, NV1020 (MediGene, Inc) was originally developed for vaccine use and derives from R7020. One copy of the $\gamma_1$34.5 gene is deleted in this virus and an HSV-2 DNA fragment encoding glycoproteins D, G, I, and portion of E is inserted in place of the internal inverted repeat (Meignier *et al.*, 1988). Since one copy of the ICP34.5 gene is intact, R7020 replicated to much higher levels compared to the ICP34.5 double-deletion mutant R3616 both *in vitro* and *in vivo* in animal tumors (Advani *et al.*, 1999). Furthermore, it had a significantly better antitumor effect than R3616 or G207 (McAuliffe *et al.*, 2000; Cozzi *et al.*, 2001; Bennett *et al.*, 2002; Chung *et al.*, 2002; Cozzi *et al.*, 2002). This vector has been tested widely in preclinical tumor models, as well as in monkeys and in humans demonstrating safety and efficacy (Meignier *et al.*, 1990; McAuliffe *et al.*, 2000; Cozzi *et al.*, 2001; Wong *et al.*, 2001a; Ebright *et al.*, 2002; Kemeny *et al.*, 2006).

HF10, a spontaneously generated mutant of HSV-1 strain HF, is another oncolytic vector used in clinical trials in Japan (Nakao *et al.*, 2007). It has a deletion of UL56 and duplications of UL53, UL54 and UL55 (Takakuwa *et al.*, 2003).

Several other HSV-1 mutants with multiple gene deletions have been engineered and evaluated. G47Δ (MediGene, Inc) is a triple-mutated vector derived from G207. It was constructed by deleting the gene encoding ICP47 (α47 or US12) from G207 (Todo *et al.*, 2001). ICP47 inhibits MHC class I antigen presentation on the surface of infected cells by inhibiting the transporter associated with antigen presentation (TAP) (York *et al.*, 1994). This leads to increased MHC class I expression, promoting tumor antigen presentation and antitumor immunity. This virus was more efficacious than its parental G207 virus *in vivo* when tested in both immunocompetent and immunodeficient animal models (Todo *et al.*, 2001).

Multiple HSV-1 oncolytic strains have been engineered by utilizing tumor-specific cellular promoters. Myb34.5 (Chung *et al.*, 1999) has shown great tumor specificity (Nakamura

et al., 2002). This mutant has both of the endogenous copies of $\gamma_1$34.5 deleted and this gene is reinserted into the ICP6 locus under control of B-myb promoter. Compared to hrR3, Myb34.5 is more attenuated in normal cells both *in vitro* and *in vivo*. It was less toxic in mice after intravenous administration. Cytotoxicity of Myb34.5 in tumor cells, however, was as robust as that of hrR3 (Nakamura *et al.*, 2002). DF3γ34.5 is a similar virus, in which expression of ICP34.5 is controlled by DF3/MUC promoter/enhancer sequence (Hinoda *et al.*, 2003). This virus has shown preferential replication in tumors that express DF3/MUC, restricted biodistribution, and reduced toxicity after systemic administration (Mullen *et al.*, 2002; Kasuya *et al.*, 2004). Several other viruses have been constructed in which essential HSV-1 immediate-early genes are under the control of CEA promoter (Mullen *et al.*, 2002), albumin promoter (Miyatake *et al.*, 1997; Miyatake, 2002), etc.

Another approach, vector re-targeting, has been explored extensively as a unique strategy to increase tumor specificity of oncolytic adenoviral vectors (Everts and Curiel, 2004). Several studies have demonstrated the feasibility of restricting HSV-1 infection to predefined subpopulations of cells (Burton *et al.*, 2002). However, there is only one truly tropism-modified oncolytic HSV-1 vector reported. The vector R5141 targets IL-13 receptor-positive glioma cells, in which the viral glycoproteins C and D are engineered as a chimeric protein fused with the IL-13 peptide enabling the virus to enter exclusively via the IL-13 receptor (Zhou and Roizman, 2006).

To enhance the efficacy of oncolytic viruses, they are often "armed" with genes that augment their cytolytic capacities. Oncolytic HSV-1 vectors have been used successfully to deliver immunoregulatory molecules, prodrug converting enzymes and angiogenesis inhibitors (Mullen *et al.*, 2004). Studies showed synergism when oncolytic HSV-1 mutants were engineered to express cytokines. It was demonstrated that cytokine expression may be an

important adjunct to oncolytic HSV-1 therapy. IL-4 which is an immunostimulatory cytokine and IL-10 which is an immunosuppressive cytokine were separately inserted in HSV-1 that had both copies of $\gamma_1$34.5 deleted. In an immunocompetent mouse glioma model, inoculation of these vectors produced opposite physiologic responses. The first vector expressing IL-4 significantly prolonged survival of tumor bearing mice compared to the parental virus, whereas mice that were inoculated with the IL-10-expressing virus had a median survival identical to that of saline-inoculated controls (Andreansky *et al.*, 1998). Ino and colleagues compared vectors expressing IL-12, IL-18, or B7-1. They reported that the IL-12 expressing vector showed significantly greater antitumor efficacy compared with other vectors (Ino *et al.*, 2006). Other groups compared an IL-12-expressing vector (NV1042) with a GM-CSF-expressing vector (NV1034) and a control vector with no transgene (NV1023), and showed that NV1042 was significantly more efficacious than NV1034 or NV1023 (Wong *et al.*, 2001b; Varghese *et al.*, 2006). It was also suggested that the combination of IL-12, IL-18, and B7-1 transgene expressing vectors might be more efficacious in inducing antitumor immunity (Ino *et al.*, 2006). Another immunomodulatory transgene-expressing vector, OncoVEX GM-CSF (BioVex Ltd, UK) is an oncolytic HSV-1 vector carrying human GM-CSF. This virus has been used in phase II clinical trials for the treatment of malignant melanoma and head and neck cancer (Senzer *et al.*, 2007).

To date, several prodrug converting enzymes have been used as transgenes inserted into HSV-1 vectors. In HSV1yCD viral UL39 gene is disrupted by sequences coding yeast cytosine deaminase (yCD), which metabolizes the prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). In mice with diffuse liver metastases this virus was effective and prolonged animal survival (Nakamura *et al.*, 2001). The CYP2B1 gene encodes the rat liver-specific enzyme cytochrome P450(CYP)2B (Chase *et al.*, 1998). This enzyme is involved in the metabolism of many drugs, and converts prodrug cyclophosphamide (CPA) into an active form. An oncolytic agent rRp450

was constructed by inserting the CYP2B1 gene cassette in place of the ICP6-LacZ fusion gene in the parental hrR3 virus, expressing the gene under the viral ICP6 promoter (Chase *et al.*, 1998). The killing of tumor cells with rRp450 and CPA was significantly more efficacious than with either rRp450 alone, hrR3 with CPA, or hrR3 alone. The combination of rRp450 and CPA was also more efficacious in vivo (Chase *et al.*, 1998).

Several oncolytic HSV-1 vectors expressing anti-angiogenic peptides have been reported: endostatin (Mullen *et al.*, 2004), angiostatin-endostatin fusion protein (Yang *et al.*, 2005), dominant-negative FGF receptor (dnFGFR) (Liu *et al.*, 2006a), platelet factor (PF4) (Liu *et al.*, 2006b). When injected into subcutaneous tumors of mice, the transgene-expressing vectors showed significantly better tumor suppression and reduced vessel density in the tumor tissues compared with the control vector (Liu *et al.*, 2006a; Liu *et al.*, 2006b).

Breast Cancer Mouse Models

Animal models have been critical in the study of the molecular mechanisms of cancer and in the development of new antitumor agents providing unparalleled insights into the field. The relevance of each particular model depends on how close it replicates the human disease, in its form and its function (Van Dyke and Jacks, 2002); the histology, physiological effects, biochemical pathways and metastatic pattern observed in the same human tumor type. The closer we get to the ideal cancer model, the more we will further understand the alterations that drive tumorigenesis. This will allow the validation of novel targets, the discovery of novel preventive or therapeutic antitumor agents and the study of drugs in vivo. Today, the mouse is the most commonly used model in the cancer research field because of our genomic and biochemical knowledge of this animal species, its extensive use and ease of manipulation. However, there is still much room for improvement in modeling cancer.

Mouse models for human breast cancer can be categorized into three main groups: (1) syngeneic and xenograft models; (2) chemically induced, virally induced, or ionizing radiation-induced models; and (3) genetically engineered mice (GEM) such as transgenics and knockouts.

Syngeneic and Genetically Engineered Models of Breast Cancer

The biology of rodents and their tumors differs significantly from that of humans and human cancer. The differences of developmental programs of mouse and humans manifest in many ways, with smaller size and shorter lifespan of mice being obvious examples. Cellular targets for oncogenic transformation differ in number, in their degree of maturation and in their differentiation in mouse tissues compared with humans. For example, full glandular maturation is contingent on pregnancy in rodents in the mammary gland, but not in humans. The shorter lifespan means that observable tumors must have a rapid program of progression. Cells of rodent origin are much easier to transform in vitro, possible explanations being the less efficient DNA repair, poorer control of genetic stability, and/or altered control of gene expression (Holliday, 1996). Furthermore, rodent cells become immortalized easier (Newbold, 1997; Greenberg *et al.*, 1998). The differences in xenobiotic metabolism, mutational pattern and genetic pathways that cause transformation between mouse and human cells translate into phenotypic differences in oncogenesis in vivo (Jacks, 1996; Wagner, 2004). Thus, whereas mice tend to develop cancer in mesenchymal tissues, human tumors derive mainly from epithelial cells. Moreover, there are functional and morphological differences between mouse tumors and the human epithelial tumors they intend to model. For instance, although similar morphological patterns can be seen in both species, mammary tumors in mice cannot be classified in an equivalent manner to standard human breast cancer pathology grades and types (Cardiff, 2001). In addition, about half of the human breast cancers are hormone responsive at diagnosis, while the majority of mouse mammary tumors are hormone independent, and have lower levels of estrogen/progesterone

receptors than human tumors (Nandi *et al.*, 1995). Furthermore, despite the fact that animal models frequently develop spontaneous tumors, which have been critical in understanding tumor initiation and primary tumor growth, they are significantly less metastatic than human tumors (McClatchey, 1999; Van Dyke and Jacks, 2002; Khanna and Hunter, 2005). This may be due to the differences in mutational rate and transformation between both species, and/or to the shorter lifespan of the mouse, which may not be sufficient for metastatic progression. Breast cancer in humans usually spreads lymphatically (starting with local lymph glands, followed by hematogenous route of distant metastasis to the bone, the brain, the adrenal gland, the liver and the lung). Mouse mammary cancers metastasize almost exclusively to the lung via the hematogeneous route (Cheung *et al.*, 1997).

Genetically engineered mice (GEM) have contributed greatly to the different aspects of studies of breast cancer disease in general. However, GEMs, like syngeneic rodent models, also present fundamental differences at the level of organism and cell. They are designed to reproduce very specific aspects of tumor formation and progression, usually based on human tumor knowledge. They do not mimic sporadic tumors that arise from an initiating mutation affecting a single cell in an otherwise normal microenvironment. To date most transgenic and knock-out mice have had a whole-body phenotype, in which all tissues and cells bear the same defect. Despite the above mentioned as well as other limitations, the syngeneic and GEM models have contributed significantly to our understanding of fundamental aspects of breast cancer genetics, but do not provide sufficient similarity with human tumors, especially for preclinical drug testing (Hahn and Weinberg, 2002).

Xenograft Models of Breast Cancer

Among the mouse models for breast cancer, xenografts still play the dominant role in preclinical trials. The most direct way to ‘humanize’ cancer animal models is to merge human

and animal models in the form of heterotransplanted tissues, implanted either heterotopically (subcutaneous) or orthotopically (mammary fat pad). Differently from the GEM models, the xenograft models allow the direct assessment of the biological behavior, or the response to drugs, of human tumors. In these models, tumor cells are human, whereas stromal cells derive from the mouse. After the discovery of the nude mouse in 1966, the discovery that the human neoplasms could be grown in nude mice was immediately recognized as an important research tool. The discovery of SCID mice in 1980 further enhanced the cancer research using mouse models (Bankert *et al.*, 2001). There are currently many xenograft models available for use in breast cancer research, most derived from established cancer cell lines and spontaneously or genetically engineered immortalized normal breast epithelial cells. Differing from the GEM models, the xenograft models allow the direct assessment of the biological behavior, or the response to drugs, of human tumors. Their reproducibility, stability, and reflection of their human origin are clearly considerable strengths.

Though there are no doubts about the nude mouse xenograft model systems having been a major breakthrough for cancer research, there are several limitations of these models, one precisely being their use of an immunocompromised host, which eliminates the possibility of studying the role of the immune system in tumor progression. The use of established cancer cell lines raises the question of them having different environmental requirements to primary breast tumor cells. Some authors also believe that cancer and host cells being from different species may limit the occurrence of critical tumor-stroma interactions, leading to inefficient signaling (De Wever and Mareel, 2003).Aslo, the murine stroma that exists in xenografts results in chimeric tumors with biology significantly different from that of humans potentially resulting in unpredictable properties (Hahn and Weinberg, 2002). In the case of orthotopic models, the organ of implantation could also become a limitation of these systems.

However, there are solutions to some of the above mentioned limitations. For example, it is now possible to grow intact primary human breast lesions and cancers in athymic mice. The dissociated cells from surgical specimens can be mixed with extracellular matrices and transplanted into mice (Yang *et al.*, 2000). Despite the potential limitations, the similarities between mouse xenograft models and clinical breast cancer are substantial. The histology of these xenografts frequently closely reflects the variety of human adenocarcinomas. Similarly, despite the severe immunodeficiencies apparent in homozygous nu/nu mice, they are remarkably robust. This is almost certainly due to their remaining immune-competence. Although nude mice lack T cells, nude mice have normal complement of bone-marrow-dependent B cells. Nude mice have elevated levels of both macrophages and NK cells, their macrophages being more potent than those from mice with normal thymus (Croy *et al.*, 2001; Meyerrose *et al.*, 2003).

Today, the mouse is the most commonly used model for the analysis of the tumorigenic process and the response of the tumor to therapies in preclinical studies. The choice of a mouse model relies on the scientist that needs to consider how close the model replicates the human disease, in its form and its function, and how well and closely could that model answer the specific questions to be addressed.

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CHAPTER II

EFFECTIVE TREATMENT OF HUMAN BREAST TUMORS IN A MOUSE XENOGRAFT MODEL WITH A HERPES SIMPLEX VIRUS TYPE-1 SPECIFYING THE NV1020 GENOMIC DELETION AND THE gBsyn3 SYNCYTIAL MUTATION ENABLING HIGH VIRAL REPLICATION AND SPREAD IN BREAST CANCER CELLS*

Introduction

Breast cancer is the most common cancer among women, excluding cancers of the skin, accounting for nearly 1 in 3 cancers diagnosed in US women (American Cancer Society, 2006). In western countries breast cancer is the second leading cause of cancer death in women and is associated with high morbidity and mortality. A new and promising strategy for cancer therapy is the use of modified viruses that have been engineered to selectively replicate within cancer cells (oncolytic virotherapy). A number of viruses have been explored as tumor-selective replicating vectors, including adenovirus, herpes simplex virus type-1 (HSV-1), vaccinia virus, reovirus, Newcastle disease virus, vesicular stomatitis virus, measles virus, poliovirus and West Nile virus (Chiocca, 2002; Kirn, 2003).

HSV-1 oncolytic strains have been increasingly explored as experimental therapeutics against a variety of cancers (Todo, 2002; Varghese and Rabkin, 2002; Mohr, 2003; Latchman, 2005; Shen and Nemunaitis, 2006). HSV-1 has a number of important advantages as an oncolytic virus including: 1) The HSV-1 DNA genome is infectious and can be easily manipulated via recombinant DNA means. 2) HSV-1 can infect most cell types and tumor lineages (wide host-range); 3) The viral genome can be manipulated to carry multiple therapeutic transgenes that can enhance its oncolytic activity; 4) Deletion of certain HSV-1 genes can restrict viral replication exclusively to cancer cells; 5) HSV-1 rarely produces life-threatening medical illness in immunocompetent adults; 6) HSV-1 replication-competent vectors have been safely

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administered in human organs including the brain of patients; 7) There are several anti-HSV-1 drugs available, which can rapidly terminate experimental infections. Over the last decade, a number of HSV-1 oncolytic viruses have been constructed and have demonstrated substantial antitumor activities in both experimental animals and humans (Todo, 2002; Varghese and Rabkin, 2002; Mohr, 2003; Latchman, 2005; Shen and Nemunaitis, 2006). These attributes render HSV-1 as one one of the most promising viral platforms for the development of improved oncolytic vectors. However, the first-generation HSV-1 vectors have shown certain limitations regarding their efficacy and safety in animal and human hosts. New generations of innovative HSV-1 vectors with improved potency and safety are required before the oncolytic strategy using HSV-1 becomes a standard therapeutic reality against cancer.

Two main genetic approaches have been used to construct HSV-1 oncolytic strains that can exclusively replicate in cancer cells: A) Deletion of the viral γ_1 34.5 gene, a major neurovirulence gene and an inhibitor of cellular apoptosis, drastically attenuates the virus and restricts viral growth to cancer cells due to the lack of intact apoptotic mechanisms in cancer cells (Chou *et al.*, 1990; Chou and Roizman, 1992). B) The HSV-1 UL39 gene codes for the large subunit of ribonucleotide reductase, an enzyme required for efficient viral DNA replication (Boviatsis *et al.*, 1994; Mineta *et al.*, 1994). This enzyme is overexpressed in most tumor cells. As a consequence, HSV-1 mutants that lack the UL39 gene preferentially replicate within tumor cells, but not within normal cells. Experiments with animal models as well as phase I/II clinical trials have revealed that viruses that have both copies of the γ_1 34.5 genes deleted do not spread very efficiently within tumors, in comparison to their parental wild-type strains (Kramm *et al.*, 1997). Another potential drawback is that the antitumor activity of oncolytic HSV does not induce a significant bystander effect (the killing of uninfected cells after death of the infected neighboring cells). These potential drawbacks have necessitated the need for construction of new HSV-1

strains that can replicate and spread efficiently within tumors. Generally, current strategies for construction of oncolytic HSV-1 strains include: 1) the deletion of only one of the two γ_1 34.5 genes allowing for improved viral replication, while the virus remains largely attenuated; 2) expression of the γ_1 34.5 gene under the control of a tumor gene promoter; 3) enhancing the ability of the virus to spread to uninfected cells via virus-induced cell fusion (Bateman *et al.*, 2000; Higuchi *et al.*, 2000).

Typically, wild-type strains of HSV induce a limited amount of cell-to-cell fusion both in vitro and in vivo. However, certain HSV-1 variants (syncytial or syn mutants) are capable of inducing extensive cell fusion (large multinucleated cells or syncytia). Our laboratory has focused and extensively published work on the structure and function of viral proteins and glycoproteins that function in virus-induced membrane fusion, virion assembly and egress (Pellett *et al.*, 1985; Baghian *et al.*, 1992; Jayachandra *et al.*, 1997; Foster *et al.*, 1998; Foster *et al.*, 1999; Foster and Kousoulas, 1999; Foster *et al.*, 2001a; Foster *et al.*, 2001b; Foster *et al.*, 2003; Foster *et al.*, 2004a; Foster *et al.*, 2004b; Melancon *et al.*, 2004; Melancon *et al.*, 2005). Syncytial mutations that cause extensive virus-induced cell fusion can arise in at least two of the glycoprotein genes: the UL27 gene, encoding glycoprotein B (gB) (Bzik *et al.*, 1984b, a; Pellett *et al.*, 1985), and the UL53 gene, coding for glycoprotein K (gK) (Bond and Person, 1984; Debroy *et al.*, 1985). Work in our laboratory has shown that gK functions as a heterodimer with the UL20 viral protein and that the UL20/gK heterodimer can interact and potentially modulate gB's functions in membrane fusion (Foster *et al.*, 2004b).

In this study, we have engineered the new prototypic HSV-1 recombinant virus HSV-1OncSyn (OncSyn). This virus has one of the two γ_1 34.5 genes, as well as adjacent sequences deleted and carries a syncytial mutation within the UL27 gene encoding gB. In addition, one of the two genomic regions coding for the latency associated transcripts (LAT) was deleted. The

OncSyn virus replicated efficiently in human breast cancer cells in cell culture yielding higher viral titers than the wild-type HSV-1(F) and parental Onc viruses. Importantly, the OncSyn virus spread substantially better in breast cancer cells than in Vero cells producing large syncytial plaques. Intra-tumor injections of the OncSyn virus within xenografts of human breast cancer cells injected into nude mice showed remarkable reduction of tumor size, and extensive necrosis of tumor cells. The results show that the constructed OncSyn virus can effectively kill tumor cells both in vitro and in vivo.

Materials and Methods

Cells, Viruses and Plasmids

African green monkey kidney (Vero) cells, human breast cancer cells (Hs578T) (Hackett *et al.*, 1977), and mouse mammary tumor cells (4T1) (Aslakson and Miller, 1992) were obtained from the American Type Culture Collection (Manassas, VA). The human breast adenocarcinoma line MDA-MB-435-luc expressing luciferase (MM4L) (Leuschner *et al.*, 2003) was kindly provided by Dr. C. Leuschner (Pennington Biomedical Research Center, Baton Rouge, LA). Vero and Hs578T cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, N.Y.), supplemented with 10% fetal calf serum and antibiotics. 4T1 cells were maintained in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. MM4L cells were cultured with Leibovitz's L-15 medium (Hyclone, Logan, UT) containing 10% FBS. These cells were cultured in tightly closed flasks in a 37°C incubator. The plasmid pYEbac102 containing the HSV-1(F) (Tanaka *et al.*, 2003) viral genome was kindly provided by Dr. Y. Kawaguchi (Tokyo Medical and Dental University, Tokyo, Japan). The plasmid pRB3410 was kindly provided by Dr B. Roizman (University of Chicago, Chicago, IL) (Meignier *et al.*, 1988). All viruses were routinely grown and titrated in Vero cells. The plasmid encoding red fluorescent

protein (pHcRed1-N1) was obtained from BD Biosciences, Clontech (Palo Alto, CA). The pEF6/V5-His-TOPO plasmid was obtained from Invitrogen (Carlsbad, CA).

Construction and Genomic Characterization of Recombinant Viruses HSV-1Onc (Onc) and HSV-1OncSyn (OncSyn)

The red fluorescent protein (RFP) gene was PCR-amplified from plasmid pHcRed1-N1 and cloned into the plasmid pEF6/V5-HisTOPO. Subsequently, the RFP gene under the elongation factor 1-alpha (EF-1α) promoter was cloned into the pRB3410 XbaI site producing plasmid pJM-R. In this plasmid the RFP gene cassette interrupts the viral sequence creating two 1838 and 2300 bp viral DNA flanking segments to facilitate homologous recombination with the viral genome.

Vero cells were transfected with pJM-R and twenty-four hours post-transfection cells were infected with the pYEbac102-derived virus. Red virus plaques formed on Vero cells were collected and sequentially plaque-purified at least six times. The resultant virus was named HSV-1Onc (Onc). An HSV-1(F) isolate constructed in this laboratory to contain a single amino acid change in glycoprotein B (gBsyn3) (Melancon *et al.*, 2005) was used in co-infection experiments with the Onc virus to isolate a virus that contained both the Onc and gBSyn3 mutations. The resultant virus (OncSyn) was plaque purified at least six times. The targeted deletions of the γ_1 34.5 gene and neighboring sequences, including the UL56, α 0, and α 4 genes, and the concomitant insertion of the HcRed gene cassette were confirmed by restriction endonuclease analysis, diagnostic PCR and sequencing. The OncSyn viral genome was recovered as a bacterial artificial chromosome into E coli using similar methodologies described in the isolation of the pYEbac102 (Tanaka *et al.*, 2003). The original pYEbac102 containing the HSV-1(F) genome was compared to pOncSyn containing the HSV-1(F) OncSyn genome via restriction EcoRI endonuclease analysis. For diagnostic PCR, primers F-UL54end (A: 5′-AGGAGTGTT

CGA GTCGTGTCT-3′) and R-ICP4prom (B: 5′-TGGGAC TATATGAGCCCGAG-3′) flanking the insertion site were used to confirm the presence of the insertion in place of the deleted genomic region. Primer A mentioned above and primer R-HcRed (C: 5′-CCTGCTGAAG GAGAGTATGCG-3′) were used to confirm the presence of the inserted HcRed gene cassette. Primers F-gK (D: 5′-ATGCTCGCCGTCCGTTCCCTGC-3′) and R-gK (E: 5′-ATCAAACAG GCGCCTCTGGATC -3′) were used to amplify the UL53 gene as a positive control.

Phenotypic Characterization and Replication Kinetics of Onc and OncSyn Viruses

Cells (both Vero and the tumor cell lines) were seeded into 6-well plates and infected the following day (when they reached approximately 95% confluency) with either Onc or OncSyn at a dose ranging from 0.1 to 0.001 PFU/cell. Cells were cultured in a maintenance medium (containing 1% FBS) and were left for 2 days to allow for the plaques and the cell fusion to develop. Photographs of the infected cells were taken using a fluorescent microscope. For plaque morphology visualized by immunohistochemistry, Vero cells were infected at MOIs of 0.01(low) and 1 (high) with HSV-1 (F), Onc and OncSyn viruses and visualized by immunohistochemistry at 48 hours pos-tinfection (h.p.i.) with horseradish peroxidase-conjugated anti-HSV antibodies (Dako, Carpinteri, CA) and Novared (VectorLabs, Burlingame, CA) substrate development. To determine the replication kinetics of the viruses, analysis of one-step growth kinetics was employed as described previously (Foster *et al.*, 2001b; Foster *et al.*, 2003). Briefly, each virus at an MOI of 2 was adsorbed to approximately 6×10^5 Vero or MM4L cells at 4°C for 1 h. Thereafter, warm medium was added, and virus was allowed to penetrate for 2 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment with phosphate buffered saline at pH 3.0. Cells and supernatants were harvested immediately thereafter (0 h) or after 6, 12, 18, 24, or 30 h of incubation at 37°C. Virus titers were determined by endpoint titration of virus stocks on Vero cells.

FACS Analysis

Monoclonal antibodies against glycoproteins gB (HSB1), gC (HSC1), and gH (52-S) were used as described previously (Cavalcoli *et al.*, 1993). A polyclonal mouse anti-gD antibody was used as described previously (Baghian *et al.*, 2002). Monolayers of Vero cells were infected with Onc and OncSyn viruses. Cells were harvested 14 h after infection and washed twice with phosphate-buffered saline (PBS) containing 2% fetal bovine serum and concentrated by centrifugation. The procedures were performed as described previously (Baghian *et al.*, 1992). Cell pellets were resuspended in 100µl of the appropriate antibody dilution, and incubated at 4ºC for 1h. Unbound antibody was removed by washing the cells twice with PBS. Pellets of cells were incubated for 1 h with 100µl of the secondary antibody, a fluorescein-conjugated goat anti-mouse antibody (ICN Pharmaceuticals, Inc., Aurora, OH), diluted 1:50 in PBS containing 10% heat inactivated goat serum and then incubated for an additional 1 h at 4ºC. The cells were washed twice as described above and analyzed immediately. A standard fluorescence-activated cell sorter (FACS) analysis was carried out with a FACScan (BD Biosciences, San Jose, CA). Images of the FACS data were generated with Cell Quest software (BD Biosciences, San Jose, CA).

Animal Experiments

Female athymic nude mice (6 weeks of age) (Charles River Laboratories, Inc, Wilmington, MA) were housed in autoclaved cages with a high efficiency filter-tops and autoclaved bedding. The animal room was kept at 25°C with a 12 hour light-dark cycle. At 6-7 weeks of age the animals (21-23g body weight) were subcutaneously implanted with 1×10^6 MM4L cells suspended in 0.1 ml of PBS and 0.3 ml of phenol red free Matrigel (Collaborative Biomedical Products, Beckton Dickinson Labware, Bedford, MA) using a 27 gauge needle (Wilson and Sinha, 1997; Koshizuka *et al.*, 1999). Body weights were determined weekly, and tumor size was

monitored beginning 16 days after tumor inoculation by measuring with a digital microcaliper. Tumor volumes were calculated using the following formula: volume = (length × width × height)/2. At an average tumor volume of approximately 70-80 mm3 (day 22 after tumor cell inoculation), the 72 experimental animals were randomized into 3 groups with two subgroups each using a randomization plan (www.randomization.com). The 3 groups of mice (24 animals per group) received 3 intra-tumoral injections of the OncSyn viral particles, PBS, or heat and UV inactivated OncSyn. Injections per tumor contained 2 x 10^6 viruses per injection in 250µl volume, 250µl of PBS, or 250µl of heat and UV inactivated OncSyn. The tumors from half of the animals from each group (one subgroup) were visualized by a chemiluminscence in vivo imaging system IVIS 50 (Xenogen, Alameda, CA). Mice were humanely euthanized five days after the last injection of the first round. The remaining animals received the second round of injections, again consisting of three consecutive injections of OncSyn, PBS, or heat and UV inactivated OncSyn. In vivo imaging was performed and mice were humanely euthanized 5 days after the last injection. Animals were given a necropsy examination after either the first or second round of injections. The weights of freshly excised tumors were determined. The primary tumor site, all lung lobes, liver, spleen, and kidneys from each animal were fixed in 10% neutral buffered formalin, trimmed, processed routinely, stained with hematoxylin and eosin (H and E) and evaluated by light microscopy. In addition to conventional evaluation of the histological slides for pathological changes, severity of tumor cell necrosis was determined on a 0 to 4 score scale (0 = no necrosis, 1 = 1% - 25% necrosis, 2 = 26% - 50% necrosis, 3= 51% - 75% necrosis, and 4 ≥ 76% necrosis).

Chemiluminescence *in vivo* imaging of live animals was performed on the scheduled days of necropsies. Specifically, tumors of 36 animals, 12 from each group, were visualized by chemiluminescence imaging and mice were necropsied after receiving the first round of

injections. The remainder of the mice were imaged and necropsied after receiving the second round of injections using a whole animal chemiluminescence in vivo imager. Mice were injected with 150 mg/kg D-luciferin intraperitoneally (approximately 100 microliters). The luciferin was diluted with phosphate-buffered saline to a final concentration of 30 mg/ml. After 15-20 minutes to allow the luciferin to be absorbed and circulate, mice were anesthetized with Avertin (2,2,2 tribromo-ethanol) at 0.4 - 0.6 mg/gm intraperitoneally. After 3 minutes, to allow for the Avertin to take effect, the mice were imaged for 5-7 minutes.

Statistical Methods and Analyses

The SAS® statistical package (Version 9.1.3) was used for all the analyses of the in vivo studies. Distributions were examined for normality using the UNIVARIATE procedure with a Shapiro-Wilk test of normality. For the repeated measures part of the analyses of tumor volumes and tumor weights, the GLM procedure was used to conduct a repeated measures design analyzed as a split-plot arrangement of treatments with TREATMENT (inactivated OncSyn, PBS, and OncSyn) and MOUSE within TREATMENT as main plot factors. Subplot factors included PERIOD (days of measurements) and TREATMENT by PERIOD interaction. When overall analyses determined significance ($p \leq 0.05$), Tukey's HSD test was used to examine pairwise differences for main effects, and pairwise comparisons of least square means with regard to interaction effects were examined with preplanned t-tests. A one-way analysis of variance was used to analyze the $\log_e$-transformed chemiluminescence imaging data for each separate necropsy group. Necropsy data (excised tumor weights) were analyzed as a one-way analysis of variance for TREATMENT effects with Tukey's HSD test used for post hoc pairwise comparisons of individual effects in the model ($p \leq 0.05$). Necrosis scores were analyzed with the Kruskal-Wallis test to determine the effect of treatment on necrosis scores. Potential differences in animal weights were analyzed with a one-way analysis of variance. Pearson correlation

coefficients within each injection and treatment were used to examine linear relationships among animal weights, tumor weights and tumor volumes. In addition, when necrosis scores were included in the variable list a Spearman correlation coefficient was used. Animal weights within each group were also examined over time with Pearson correlations.

The animal studies were approved by the institutional animal care and use committee (IACUC) of the Louisiana State University, and animal care was in compliance with the principles of laboratory animal care of the NIH, USA.

Results

Construction and Characterization of Oncolytic HSV-1 Mutant Viruses Onc and OncSyn

To attenuate HSV-1(F), but at the same time allow for efficient replication within tumor cells, a strategy was designed to delete a large portion of the HSV-1(F) genome containing one of the two alleles of the immediate early genes α 4 and α 0, as well as the virulence gene γ_1 34.5. The strategy was similar to the one previously used for the construction of the R7020 recombinant virus, in which an approximately 16 Kbp genomic region encompassing the UL56, α 0, γ_1 34.5, and α 4 genes was deleted. The deleted genomic region of the R7020 was replaced with a herpes simplex virus type-2 (HSV-2) DNA sequences coding for viral glycoproteins gD, gG, gI and a portion of gE, as well as a gene cassette expressing the viral thymidine kinase (TK) gene under the α 4 promoter control (Meignier *et al.*, 1988). In contrast to the R7020 recombinant virus, the Onc virus was designed to contain a gene cassette coding for the enhanced red fluorescence protein inserted within the deleted genomic region, while the viral TK gene remained intact in its natural genomic location (Figure 2.1). Specifically, plasmid pRB3410 containing an approximately 16Kbp fragment spanning the viral genomic site containing the γ_1 34.5 gene was modified to include the HcRed gene cassette (RFP gene under the control of the EF-1α) immediately flanked by the UL54 and α 22 genes (Figure 2.1C; see Materials and

Methods). Homologous recombination between the transfer plasmid and the viral genome in a transfection followed by infection experiment resulted in viral plaques emitting red fluorescence when observed under a fluorescence microscope.

To facilitate virus spread via virus-induced cell fusion, the OncSyn virus was isolated after double-infection of Vero cells with Onc and a HSV-1(F) laboratory strain specifying the gBsyn3 mutation. Individual viral plaques exhibiting the syncytial phenotype and emitting red fluorescence were isolated and extensively plaque-purified. Individual viruses were plaque-purified and the targeted deletion/insertion was verified by DNA sequencing of the entire genomic region bracketing the deletion/insertion, as well as by PCR analyses (Figure 2.1D). The presence of the gBsyn3 mutation was verified by DNA sequencing. Primers A and B (see Materials and Methods) flanking the insertion site amplified the predicted 3,126-bp DNA fragment from Onc (lane 1), OncSyn (lane 1), and pOncSyn (lane 1), which contain the inserted gene cassette. As expected, the HSV-1 (F) wild-type viral DNA produced no amplified product (lane 1). Amplification using primers A and C produced the predicted 1,693-bp DNA fragment confirming the presence of the HcRed gene cassette (lane 2 for Onc, OncSyn, and pOncSyn). Primers D and E amplified the predicted 1060-bp UL53 DNA fragment from all four constructs, since they all contain the wild-type UL53 gene (lane 3).

To facilitate further characterization of the OncSyn genome and the rapid construction of additional OncSyn recombinant viruses containing other transgenes of interest, the OncSyn genome was recovered in E. coli as a bacterial artificial chromosome (bac), since it contained the original bac plasmid sequences originating from pYEbac102. The recovered pOncSyn and parental pYEbac102 plasmid DNAs were subjected to restriction endonuclease analysis as means for determining overall genomic stability. EcoRI restriction revealed that the cloned OncSyn genome produced a similar restriction pattern to the parental pYEbac102 with the exception of

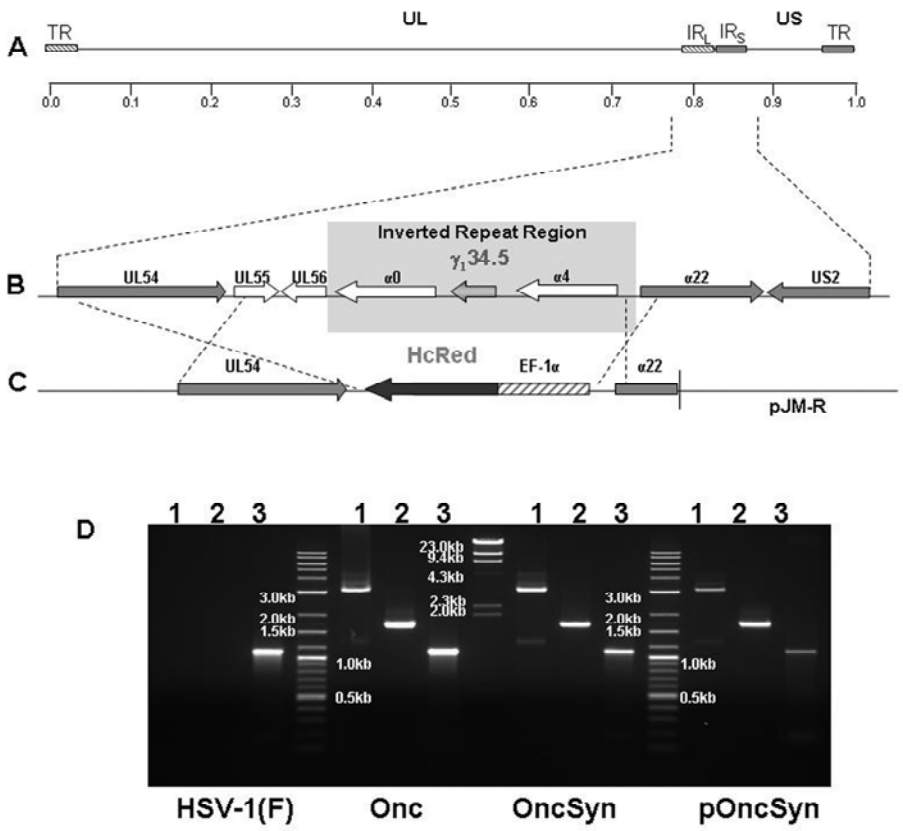

Figure 2.1. Schematic representation of the strategy used to generate the HSV-1Onc (Onc) and HSV-1OncSyn (OncSyn) viruses and PCR-based molecular characterization of the constructed viruses. (A) The top line represents the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (B) Shown below is the expanded inverted repeat region with approximate locations of UL54, UL55, UL56, α 0, γ_1 34.5, α 4, α 22 and US2 genes. (C) Schematic of the DNA fragment cloned into plasmid pJM-R, which was used for insertion of the HcRed gene cassette into the viral genome. In this plasmid, the HcRed gene cassette was inserted in place of the γ_1 34.5 gene and adjacent sequences, while other flanking sequences were retained to facilitate homologous recombination with the viral genome. (D) Diagnostic PCR test was performed using primers A and B flanking the insertion site to confirm the presence of the inserted gene cassette expressing the HcRed gene in place of the deleted genomic region in Onc (lane 1), OncSyn (lane 1) , and pOncSyn (lane 1), but not in HSV-1(F) (lane 1). The fragment sizes of the DNA ladder markers are denoted in Kbs. Primers A and C (insertion specific primer) were used to verify the specific presence of the HcRed gene in Onc (lane 2), OncSyn (lane 2), and pOncSyn (lane 2), but not in HSV-1(F) (lane 2). Primers D and E were used to amplify the UL53 gene to serve as a PCR positive control (see Materials and Methods) (lane 3 for HSV-1(F), Onc, OncSyn, and pOncSyn).

the appearance of a new diagnostic DNA fragment of approximately 8.7 Kbp produced after deletion of approximately 16 Kbp from the E+K EcoRI DNA fragment (apprx. 21.5 Kbp) and insertion of approximately 3.2 Kbp sequences containing the RFP gene cassette (Figure 2.2). A similar restriction pattern was produced by EcoRI restriction of the R7020 viral genome (Meignier *et al.*, 1988).

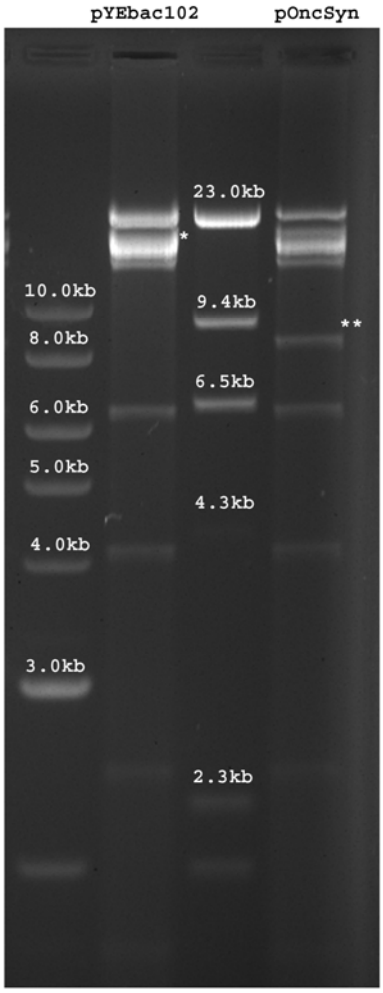

Figure 2.2. Restriction fragment analysis of pYEbac102 and pOncSyn. Photograph of an ethidium bromide-stained agarose gel in which EcoRI restricted pOncSyn and pYEbac102 DNAs were electrophoretically separated and compared. The deletion of approximately 16 Kbp originating from the E+K EcoRI DNA fragments (apprx. 21.5 Kbp; *) and insertion of approximately 3.2 Kbp sequences containing the RFP gene cassette caused the appearance of a new diagnostic DNA fragment of approximately 8.7 Kbp (**). DNA molecular mass markers are indicated above the respective DNA species.

Phenotypic and Replication Characteristics of the Onc and OncSyn Viruses

The plaque morphology of the Onc and OncSyn viruses was examined on Vero, as well as on cancer cells of different lineages including the MDA-MB-435S-luc (MM4L) (human breast tumor-derived), Hs578T (human breast tumor-derived) and 4T1 (mouse mammary tumor-derived) (see Materials and Methods).

Both viruses (Onc and OncSyn) produced plaques emitting strong red fluorescence on all cell lines tested. Importantly, both Onc and OncSyn viral plaques were substantially larger on both MM4L (Figure 2.3: C, D) and Hs578T (not shown) cells in comparison to Vero cells (Figure 2.3: A, B). To better visualize the extent of virus-induced cells fusion and syncytial plaque morphology of the OncSyn virus, Vero and MM4L cells were infected with HSV-1 (F), Onc, or OncSyn viruses at either low (0.01) or high (1) MOI, and infected cells were visualized at 48 h.p.i. by immunohistochemistry using polyclonal antibody against HSV-1 glycoproteins (Figure 2.4).

Onc and OncSyn viral plaques were substantially larger on both MM4L (Figure 2.4: I, K) and Hs578T (not shown) cells in comparison to Vero cells (Figure 2.4: C, E). In contrast, viral plaques on 4T1 cells (breast cancer cells of mouse origin) were drastically smaller (apprx. 10-fold smaller) than those produced on MM4L cells (data not shown). In contrast to the Onc virus, the OncSyn virus produced syncytial plaques, which were larger in MM4L cells than on Vero cells (Figure 2.4: K, E). Extensive cell fusion was produced by infection with OncSyn virus at high MOI characterized by large syncytia formed in the entire cell culture dish (Figure 2.4: F, L).

The immunohistochemistry results revealed that the Onc and OncSyn viruses appeared to express similar amounts of viral glycoproteins detected by the anti-HSV-1 polyclonal serum (Figure 2.4). To further assess whether individual viral glycoproteins were expressed on Onc and OncSyn-infected Vero cells, the expression of the major glycoproteins gB, gC, gD and gH was

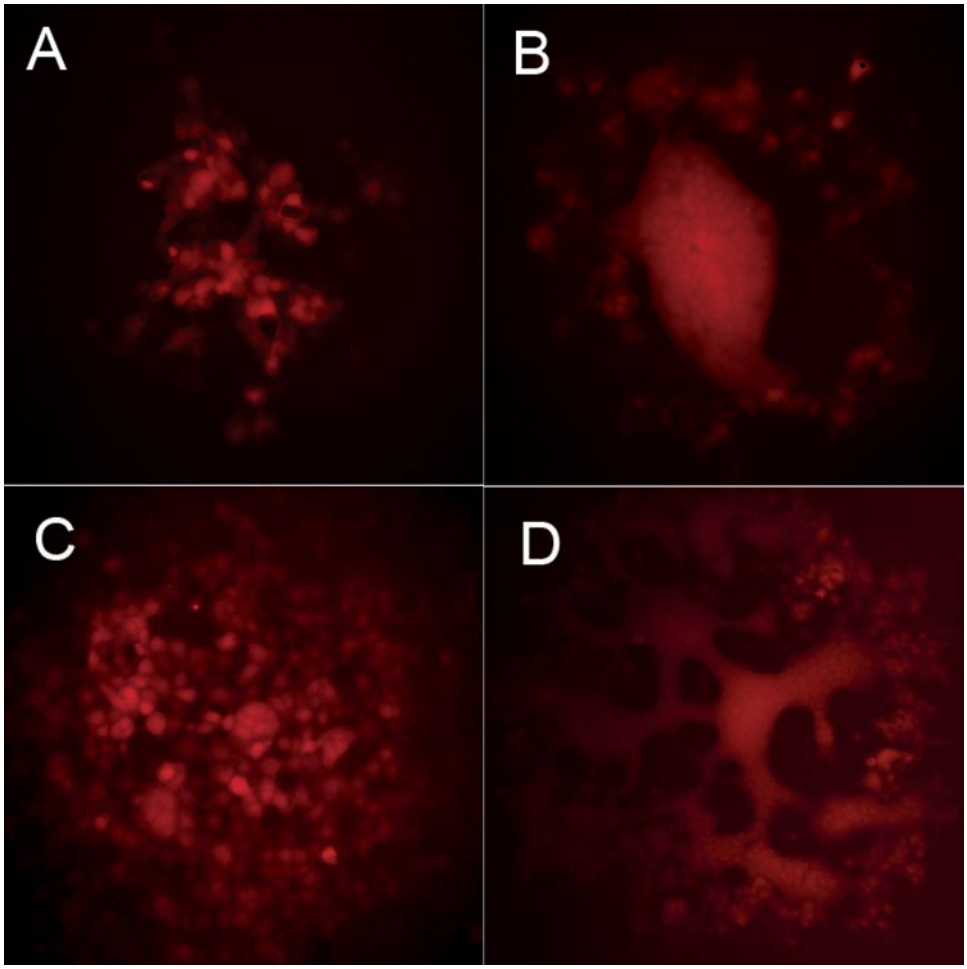

Figure 2.3. Plaque morphology of the Onc and OncSyn viruses. Vero (A, B) and MDA-MB-435S-luc (C, D) cells were infected with either Onc (A, C) or OncSyn (B, D) viruses. Individual viral plaques were photographed at 48 h.p.i. using a fluorescence microscope at 100X magnification.

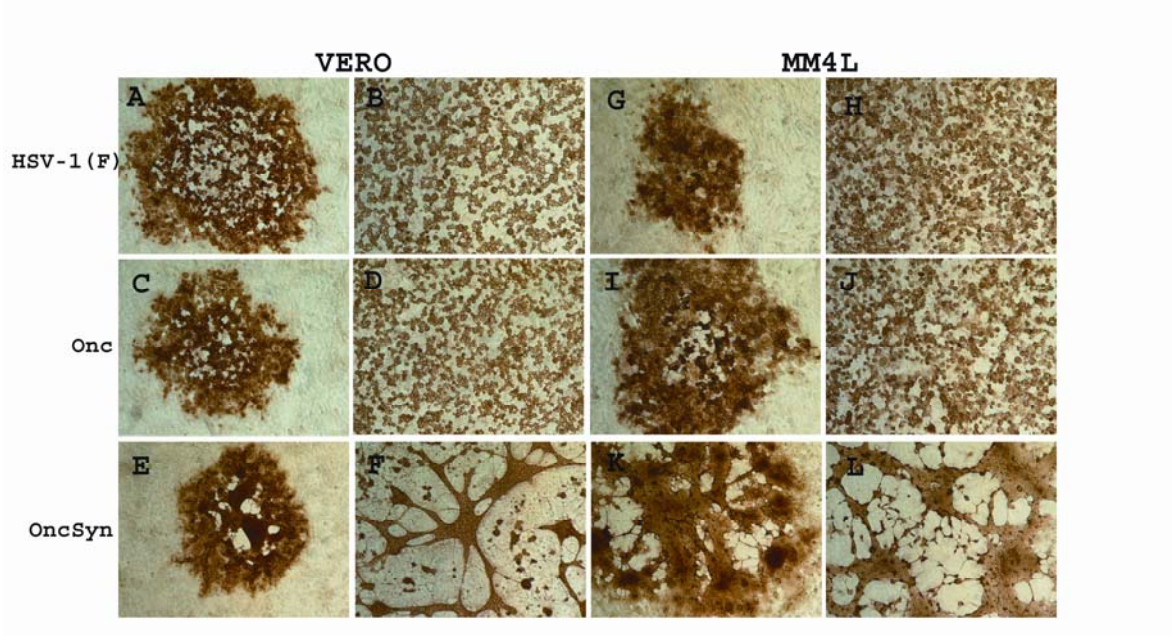

Figure 2.4. Plaque phenotypes of HSV-1(F), Onc, and OncSyn viruses. The plaque phenotypes of HSV-1(F), Onc, and OncSyn viruses were observed on Vero (A to F) and MM4L (G to L) cells. Near confluent cell monolayers were infected with wild-type HSV-1(F) derived from pYEbac102 (A, B, G, and H), Onc (C, D, I, and J), and OncSyn (E, F, K, and L) at low (A, C, E, G, I, and K) and high (B, D, F, H, J, and L) MOI. Viral plaques were visualized at 48 h.p.i. by immunohistochemistry.

detected by immunofluorescence cytometry. Individual fluorescent cytometric measurements were performed using monoclonal antibodies specific for glycoproteins gB, gC, gD, and gH as described in Materials and Methods. These experiments revealed that all viral glycoproteins were detected on infected cell surfaces and that the gBsyn3 mutation did not appear to affect gB expression (Figure 2.5).

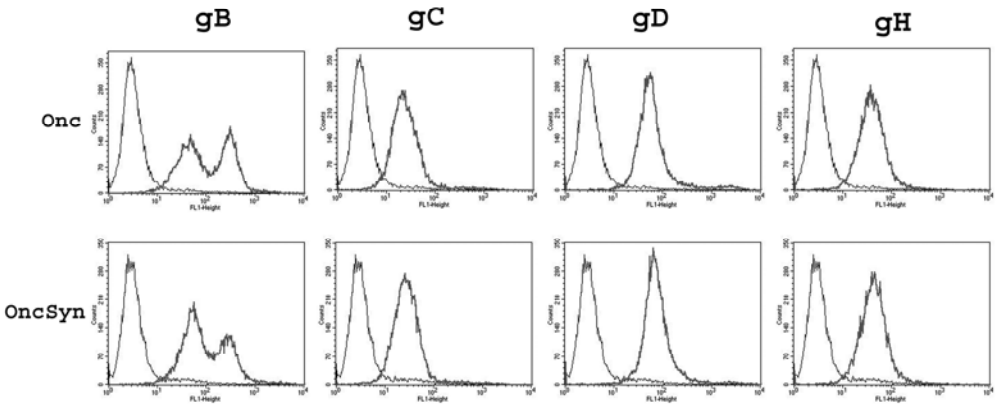

Figure 2.5. FACS analysis of the expression of glycoproteins B, C, D, and H in Vero cells infected with Onc and OncSyn viruses. Infected Vero cells were reacted with anti-gB, anti-gC, anti-gD, and anti-gH antibodies and FITC-conjugated secondary antibodies as described in Materials and Methods. The first fluorescence intensity peak represents mock-labeled control cells, while the shifted peaks immediately adjacent to the mock peak represent the antibody-labeled cells.

The time-dependent replication characteristics of the HSV-1 (F), Onc and OncSyn viruses were examined in Vero and MM4L cells infected at an MOI of 2. The OncSyn virus replicated to higher titers than the parental HSV-1 (F) and Onc viruses in MM4L cells, while OncSyn produced similar titers to the HSV-1(F) virus, but higher titers than Onc in Vero cells (Figure 2.6: A, B) . Replication of both Onc and OncSyn viruses in 4T1 cells was reduced by more than 2-logs in comparison to MM4L cells (not shown).

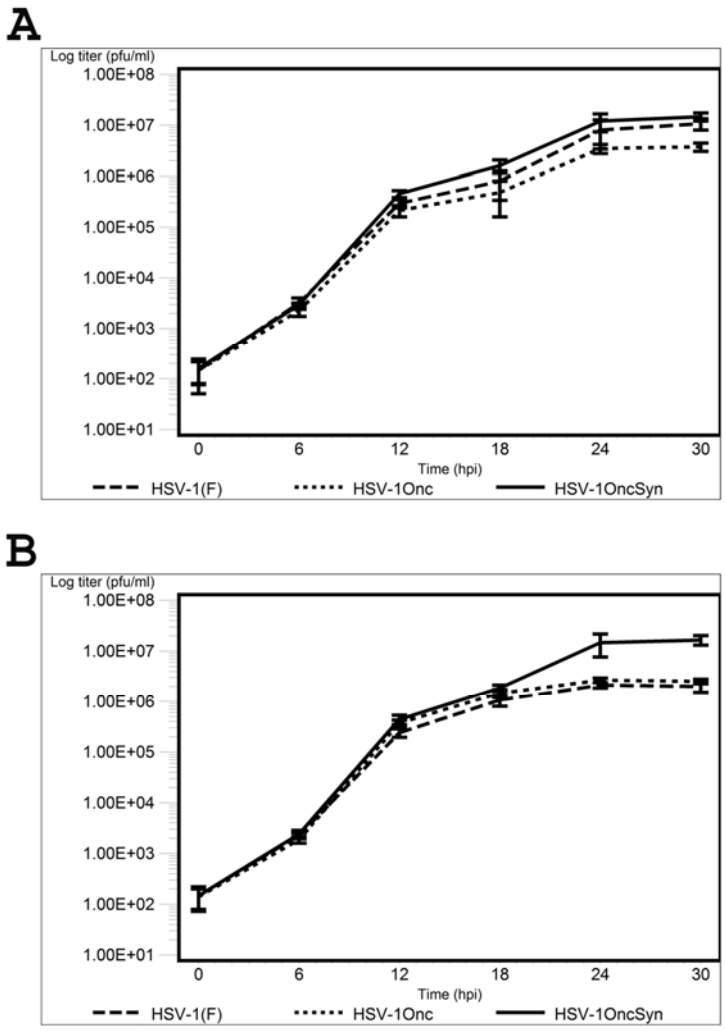

Figure 2.6. Infectious virus production on Vero and MDA-MB-435S-luc (MM4L) cells. Comparison of the viral replication kinetics of HSV-1(F), Onc, and OncSyn viruses on Vero (A) and MM4L (B) cells. One-step kinetics of infectious virus production were calculated after infection of near confluent monolayers of Vero and MM4L cells at an MOI of 2 followed by incubation at 37°C. Viral titers are shown in logarithmic scale. The error bars represent means ± 2 standard errors.

Testing of the Oncolytic Properties of the OncSyn Virus in a Xenograft Nude Mouse Model

The human breast cancer cell line MDA-MB-435S (MM4) was isolated from a human ductal carcinoma from a pleural metastatic site and has served as a model for numerous anti-tumor studies (Caillou, 1978). The MM4L cell line constitutively expresses the luciferase gene from the firefly (*Photinus pyralis*). This cell line was previously shown to facilitate visualization of tumor formation and metastasis in a xenograft mouse model (Leuschner, 2003; Leuschner and Hansel, 2004; Leuschner *et al.*, 2006). In this mouse model, MM4L cells are injected into the interscapular region of mice with Matrigel, which promotes efficient tumor establishment (Fridman *et al.*, 1991; Mehta *et al.*, 1993; Bao *et al.*, 1994). Vascularization of the primary tumor is typically observed within 10 days after tumor cell inoculation.

Subcutaneous tumors were established in female athymic Hsd-nu nude mice. When the average volume of tumors reached 70-80 mm^3, mice were randomly assigned to groups (n=24) and were treated with intratumor injections of OncSyn virus, PBS, or heat and UV inactivated OncSyn virus at multiple sites of the tumors on days 22, 25, and 28 after tumor cell inoculation. Half of the mice (n = 12) in each group were humanely sacrificed 5 days after the last injection. The remaining animals in each group (n = 12) received a second round of 3 consecutive injections at days 34, 37, and 40. These animals were humanely sacrificed 5 days after the last injection.

Tumors inoculated with a double round of OncSyn virus exhibited a time-dependent decrease in tumor size with maximum reduction of more than 80% of controls when observed at 22 days post first injection with the virus (Figure 2.7). Prior to the virus injections tumor sizes of the test group were similar to those of the two control mouse groups injected with either PBS ($p = 0.90$) or inactivated OncSyn virus ($p = 0.84$). Tumors of mice injected only with one round of

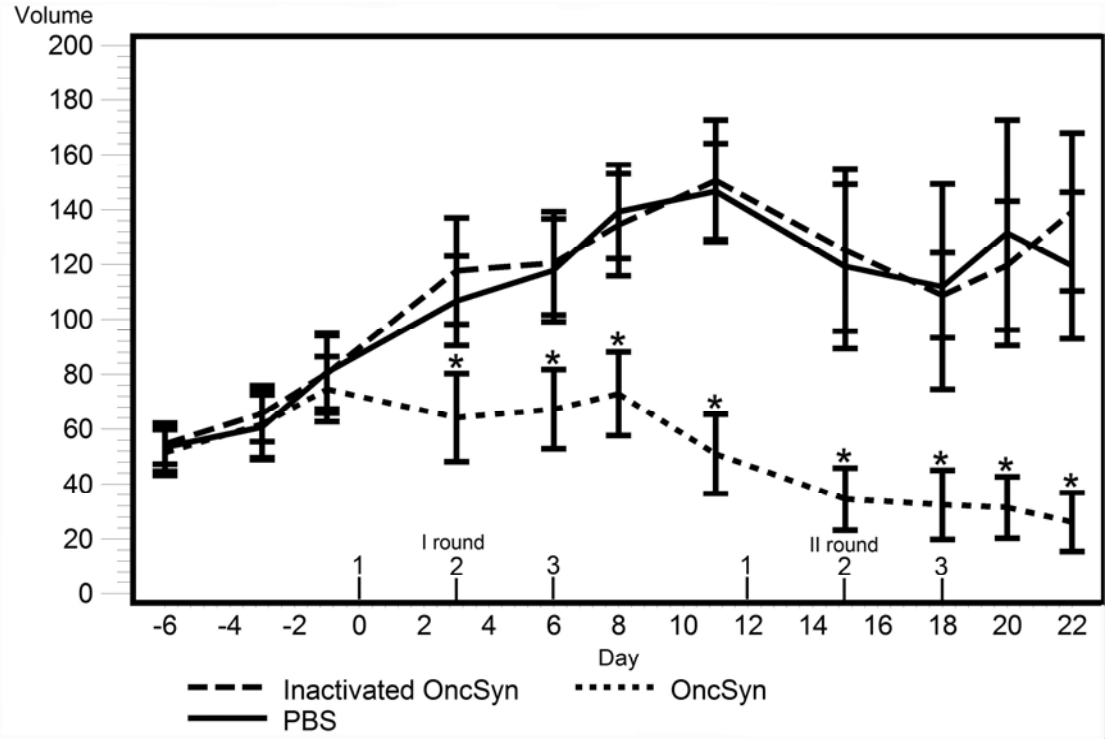

Figure 2.7. Effect of treatment on tumor volumes. Human breast tumor xenografts were established by injecting MM4L cells as a Matrigel suspension subcutaneously into the interscapular regions of mice. Tumors were measured using a digital caliper at defined time intervals prior and after treatment (x-axis). Groups of mice were treated with OncSyn, inactivated Syn, or PBS. OncSyn, inactivated OncSyn, or PBS were injected intra-tumorally when tumors reached approximately 70-80mm^3 volume. Tumor volumes were measured prior to (negative values) and after the injections. 0 on X axis represents the day of the first injection. The vertical lines on X axis represent the injection days for I and II rounds of injections (3 consecutive injections per round). At day 11 after the first injection, half of the mice (12) in each group were sacrificed. The remaining mice received a second round of three injections and were sacrificed five days after the last day on injection (day 23). The tumor volumes were determined from the formula: volume = (length × width × height)/2. The error bars represent means ± 2 standard errors. Within a given day of measurement, significant differences ($p \leq 0.05$) are indicated with an asterisk (*).

virus injections exhibited a rapid reduction of tumor size at three days after the first injection (Figure 2.7).

When compared statistically at each period to both the inactivated OncSyn group and the PBS group, the OncSyn group showed highly significant ($p < 0.0001$) reductions in tumor size from this period until the end of the study. The efficacy of treatment was not influenced by differential weight gain/loss in different mouse groups, since analysis of mouse weights during the course of the study showed no significant differences among the three groups over time. Furthermore, this analysis was corroborated by correlation analysis which indicated consistent weight gain patterns across all three study groups (not shown).

To further substantiate the oncolytic effect of the OncSyn virus on breast tumors implanted in nude mice, tumor progression and cellular viability was evaluated by chemiluminscence imaging. A total of eight mice per group either after the first or second round of viral treatment were evaluated for the presence of chemiluminescent MM4L cells. OncSyn-treated mice (Figure 2.8: C, D) exhibited drastic reduction in chemiluminescence signals emitted from tumor injection sites in comparison with the PBS and inactivated virus control injections (Figure 2.8: A, B, respectively). Similar results were obtained for OncSyn-treated mice receiving two rounds of injections (not shown). The chemilumiscence signals were quantified using relative light counts detected by the equipment. Comparison of these measurements revealed a decrease of up to 90% of light counts for the OncSyn virus-treated animal tumors compared to the control PBS or inactivated virus treated groups for both the single ($p = 0.0002$) or double ($p < 0.0001$) rounds of injections (Figure 2.9).

Pathological Examination of Tumors and Internal Organs

Individual tumors were removed, fixed for histopathological examination, and evaluated by blind observation with regard to the level of observed necrosis, presence of inflammatory

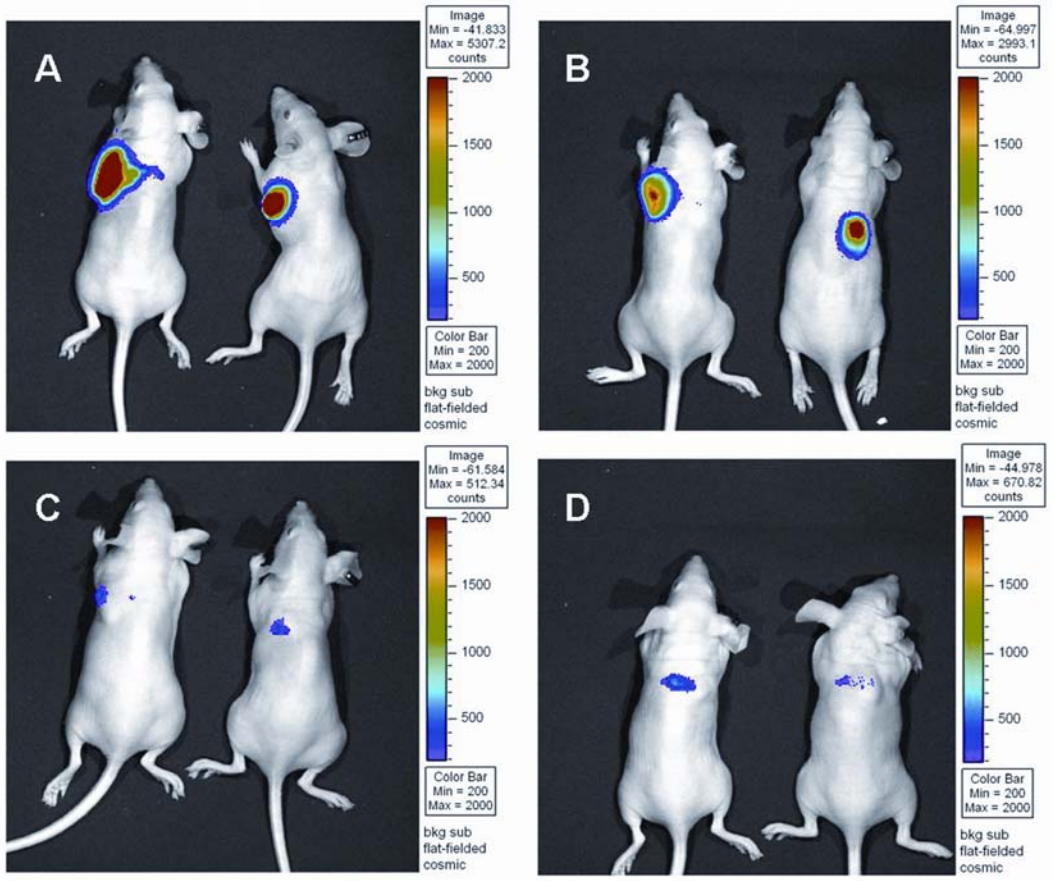

Figure 2.8. Chemiluminescence imaging of MM4L tumors in living female nude mice. Each image was produced after a 5-7 min exposure starting at 20 min following intraperitoneal injection of D-Luciferin (150mg/kg of body weight). The images of 8 animals are shown. The signal intensity in the region of interest (ROI) is expressed as light counts. Tumors were treated either with inactivated OncSyn (A), PBS (B) or OncSyn (C, D). Pictures shown represent animals after the first round of injections consisting of 3 intratumor injections of the OncSyn viral particles, PBS, or heat and UV inactivated OncSyn. Injections per tumor contained 2 x 10^6 viruses per injection in 250µl volume, 250µl of PBS, or 250µl of heat and UV inactivated OncSyn. Color bars show the scale of minimum and maximum light intensities depicted on the figure.

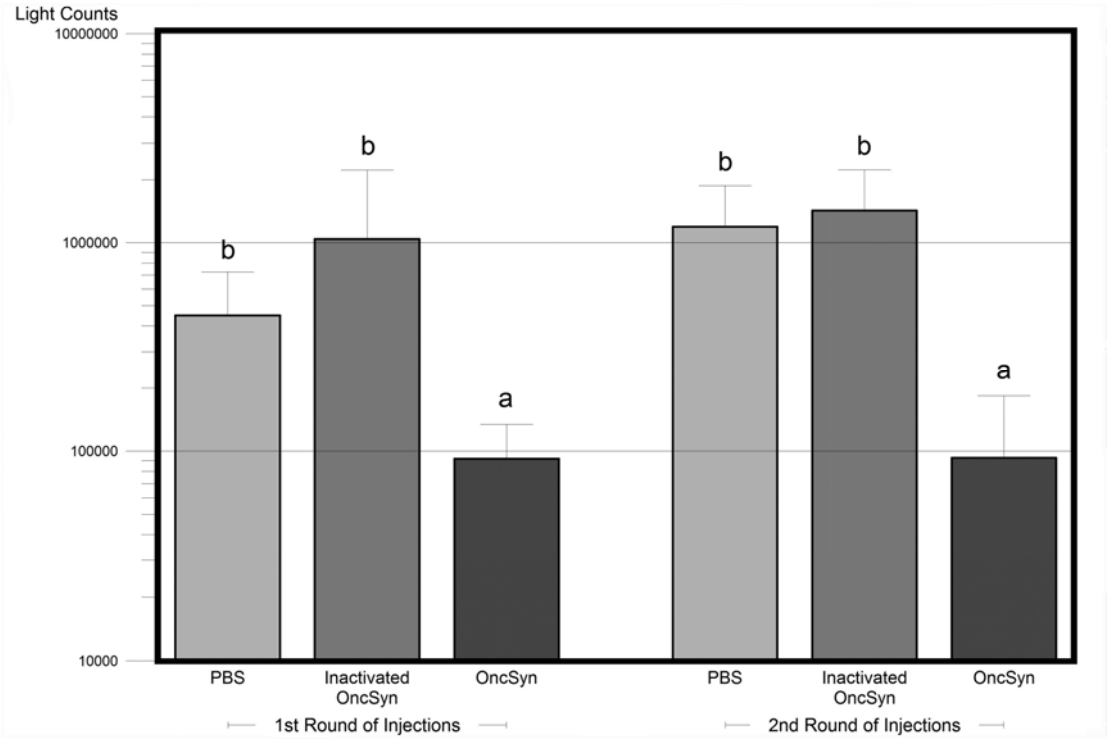

Figure 2.9. Graphic representation of relative light intensities of chemiluminescent mouse tumors. Light intensity was measured in a chemiluminscence whole animal in vivo imaging system (IVIS 50). Relative light intensities from the areas of interest were quantified for PBS, inactivated virus, or OncSyn virus treated animal tumors after the first and second rounds of injections. Light counts are set in logarithmic scale. The error bars represent 95% confidence limits. Means with the same letter in common are not significantly different ($p > 0.05$) within each round of injections.

cells, and any other abnormal morphological effects. Typically, most tumors had a connective tissue capsule formed around the tumor. In addition, vascular invasion into the tumor occurred from the connective tissue capsule. OncSyn-treated tumors had a high level of necrotic MM4L cells (Figure 2.10).

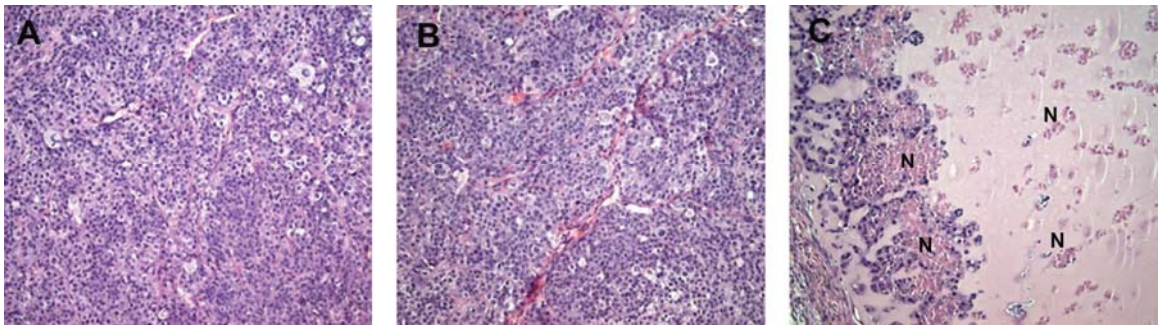

Figure 2.10. Histopathology of MM4L tumors. Mouse tumors were treated by three consecutive injections (one round), and mice were sacrificed 5 days after the last injection. Tumor-derived tissues were stained with H and E and examined. Representative stained sections are shown for inactivated OncSyn (A), PBS (B), and OncSyn (C). Panels A: Viable tumor cells are throughout the histosection. Panel B: Viable tumor cells are throughout the histosection. Panel C: Viable tumor cells are greatly reduced in number and are admixed with necrotic tumor cells (N). Also visible are necrotic tumor cells (N) within the matrigel matrix (homogenous pale eosinophilic material). All photographs were taken at 200X magnification.

The approximate amount of tumor cell necrosis was determined for each tumor histo-section. A grading scale of 1- 4 was used with 4 being the highest. The OncSyn virus caused a significant increase in necrotic tumor cells after either the single ($p < 0.0001$) or double ($p = 0.0002$) treatment with the OncSyn virus (Figure 2.11). After the single treatment, there were also highly significant negative correlations between scores and tumor weights ($r = -0.80$, $p < 0.0001$) and tumor volumes ($r = -0.68$, $p < 0.0001$). After the double treatment these values were again significant with $r = -0.63$ ($p < 0.0001$) and $r = -0.57$ ($p = 0.0004$), respectively. Microscopic examination of all lung lobes, liver, spleen, and kidneys of all the animals did not reveal any abnormalities.

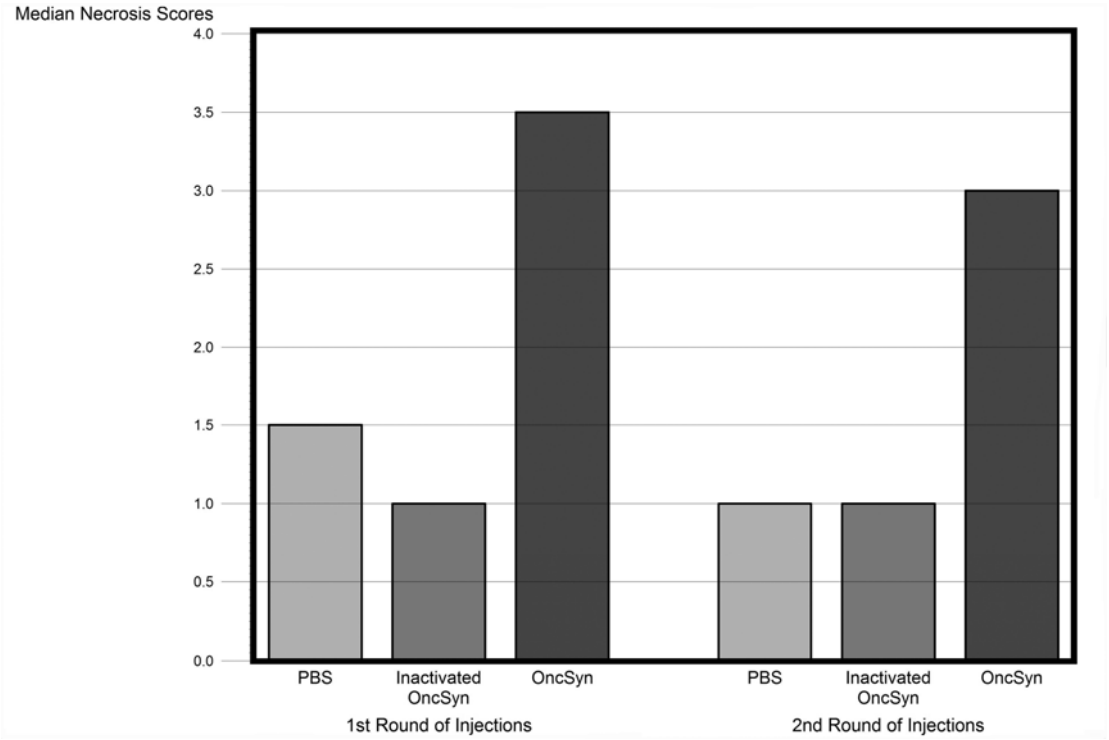

Figure 2.11. Median tumor necrosis scores determined by histopathological examination. The median values of necrosis scores were determined for samples obtained after one and two rounds of treatment with inactivated OncSyn virus, PBS, or OncSyn virus. Tumor necrosis scores were graded on a 0 to 4 score scale (see Materials and Methods).

Discussion

A number of different HSV-1 recombinant viruses have been constructed and evaluated for their ability to treat a variety of different cancers in animal models, as well as in human phase I/II clinical trials (Todo, 2002; Hu and Coffin, 2003; Argnani *et al.*, 2005; Shen and Nemunaitis, 2006). The most important modification, which is common to all constructed HSV-1 viruses is the modification/deletion of the γ_1 34.5 gene, based on the knowledge that γ_1 34.5 is an important determinant of neurovirulence and an inhibitor of cellular apoptosis. In the present investigations, we constructed and characterized the recombinant virus OncSyn that specifies the R7020 genomic deletion without the insertion of the HSV-2 DNA sequences found in the R7020. In addition, based on a series of published results with other Oncolytic HSV-1 strains that were modified to cause extensive virus-induced cell fusion, OncSyn was engineered to specify the gBsyn3 mutation, which is known to cause enhanced virus-induced cell fusion (Bzik *et al.*, 1984a; Pellett *et al.*, 1985). The salient features of our results are: 1) The OncSyn virus replicated to high titers in Vero and especially in human breast cancer cells and caused extensive virus-induced cell fusion; 2) The OncSyn virus drastically reduced tumor sizes after a single round of direct intratumor injections of human breast tumors established in nude mice; 3) The OncSyn genome was recovered as a stable bacterial artificial chromosome to enable future rapid modifications; 4) Pathological evaluation of tumor tissues revealed extensive necrosis after a single round of virus injections. These results suggest that the OncSyn virus could effectively treat breast and potentially other types of tumors and form the basis for generation of additional recombinant viruses that could be effectively used for human cancer treatment.

Oncolytic Viruses Carrying Deletions of the γ_1 34.5 Gene

Although γ_1 34.5 is non-essential for virus replication, deletion of both copies of the γ_1 34.5 genes causes substantial reduction in infectious virus production (McKie *et al.*, 1996;

Andreansky *et al.*, 1997; Kramm *et al.*, 1997; Todo *et al.*, 2001). This has led to the hypothesis that lower or conditional expression of the γ_1 34.5 may provide sufficient attenuation without substantially compromising replication efficiency. This concept has been amply demonstrated in the comparison of the previously constructed R3616 and R7020 that have either both or one of the two γ_1 34.5 deleted, respectively. R7020 replicated to much higher levels compared to the double-deletion mutant R3616 both in vitro and in vivo (Advani *et al.*, 1999). Furthermore, R7020 replicated preferentially in neoplastic cells and exhibited a remarkable safety profile in extensive rodent and primate studies as well as in human vaccine trials (Meignier *et al.*, 1988; Meignier *et al.*, 1990). R7020 is being currently evaluated in human clinical trials under the name NV1020. Recently, it was shown that NV1020 could be safely administered into the hepatic artery without significant effects on normal liver function in a phase I, open-label, dose-escalating study with subjects having metastatic colorectal carcinoma to the liver (Kemeny *et al.*, 2006). The G207 virus carries a double-deletion of the γ_1 34.5 gene, as well as a deletion of the UL39 gene coding for the large subunit of the ribonucleotide reductase. G207 has been extensively studied in animal models and human phase I/II trials. Direct comparison between G207 and NV1020 revealed that NV1020 replicated more efficiently than G207 and exhibited higher oncolytic effectiveness at lower viral doses (McAuliffe *et al.*, 2000; Cozzi *et al.*, 2001; Bennett *et al.*, 2002). Furthermore, G207 was noted to be not only attenuated for pathogenicity, but also for tumor cell killing capability (McAuliffe *et al.*, 2000; Cozzi *et al.*, 2001; Bennett *et al.*, 2002).

The OncSyn Virus

The OncSyn virus was constructed largely based on the findings that NV1020 (the clonal derivative of R7020) was sufficiently attenuated, while it maintained ample ability to replicate in vitro and in vivo (Bennett *et al.*, 2002; Cozzi *et al.*, 2002; Ebright *et al.*, 2002). The OncSyn

virus has some similarities, as well as substantial differences in comparison to NV1020. Both OncSyn and R7020 viruses are derived from the HSV-1(F) strain, with the exception that OncSyn virus was derived from the HSV-1(F) genome cloned into a bacterial artificial chromosome (pYEbac102) (Tanaka *et al.*, 2003). It has been reported that the pYEbac102-derived virus exhibited similar virulence profile to the parental HSV-1(F) strain in intracerebral infections (Tanaka *et al.*, 2003). However, immunocompetent Balb/c mice infected with the pYEbac102-derived virus via the intraperitoneal route (i.p.) did not exhibit any neurological symptoms suggesting that the pYEbac102-derived virus may be attenuated (not shown). Extensive restriction analysis and DNA sequencing of the pYEbac102-derived virus did not reveal any significant genomic changes, suggesting that either the insertion of the bac (bacterial artificial chromosome) backbone or other nucleotide changes may potentially contribute to the observed attenuation characteristics (unpublished observations). Both NV1020 and OncSyn contain a large deletion of approximately 16 kilobase-pairs (Kbp) across the joint region of the long-L and short-S components of the viral genome. This deleted region includes the UL56 gene, and one of the two copies of α 0, γ_1 34.5 and α 4 genes. In addition, this deletion also includes the entire genomic region coding for one of the two loci encoding the latency-associated transcripts (LAT). The NV1020 virus contains within the deleted genomic region a 5.2 Kbp DNA fragment of HSV-2 and an exogenous copy of the HSV-1 viral thymidine kinase (TK) under the control of the α4 promoter (the native TK gene has been deleted). In contrast, the OncSyn virus contains within the deleted genomic region an insertion of a gene cassette coding for the red fluorescence protein under the constitutive control of the EF-1α promoter, while the native TK gene remains unaltered. The presence of both HSV-1 and HSV-2 glycoproteins gD, gI and gE in the NV1020 virus may lower the relative efficiencies of intracellular virus assembly and egress and result in virus attenuation and decreased intra-tumor spread (Hu and Coffin, 2003). Alternatively, it is

possible that the presence of the HSV-2 viral glycoproteins, especially gD and gE/gI may broaden the host-range of the recombinant virus.

The OncSyn virus was constructed to specify the gBsyn3 mutation, which is known to cause extensive virus-induced cell fusion (Bzik *et al.*, 1984a; Pellett *et al.*, 1985). Infection of a variety of breast cancer cells, as well as other cell lines such as Vero, HeLa, etc., revealed that OncSyn caused extensive virus-induced cell fusion. Apparently, the syncytial phenotype provided a significant replication advantage for the virus, since the OncSyn titers were higher than those of the parental Onc virus in all cells tested. Surprisingly, the OncSyn virus produced higher viral titers than the HSV-1(F) prototypic virus in MM4L cells indicating that the increased virus spread caused by the gBsyn3 mutation substantially overcame the reduced plaque phenotype caused by the NV1020 genomic deletion. In addition to the γ_1 34.5 important anti-interferon functions, the γ_1 34.5 protein is known to function in virus maturation and egress from infected cells, as well as glycoprotein synthesis and cell-surface expression (Brown *et al.*, 1994; Mao and Rosenthal, 2003; Jing *et al.*, 2004; Jing and He, 2005). Therefore, the gBsyn3 mutation may reverse these defects by allowing more efficient virus intracellular transport and egress. It is possible that the observed increased virion egress and virus-induced cell fusion caused by the gBsyn3 mutation requires the expression of the single γ_1 34.5 allele. Construction of the gBsyn3 and other syncytial mutations in the G207 genomic background would resolve this issue.

The remarkable replication and virus-induced cell fusion properties of the OncSyn virus may cause efficient spread intratumorally. Furthermore, fusion of tumor cells may induce anti-tumor specific immunity due to improved presentation of cancer antigens (Nakamori *et al.*, 2004). Unfortunately, the OncSyn virus replicated very inefficiently in 4T1 cells preventing the further evaluation of the virus under ideal replication conditions in an immunocompetent mouse model system using 4T1 or other mouse-derived cells (not shown). Similar defects of viral

replication and virus spread have been described in the literature for other oncolytic herpesviruses (Thomas and Fraser, 2003). Similar results have been obtained with other HSV-1 based oncolytic viruses containing syncytial mutations of unspecified origin (Fu and Zhang, 2002), as well as other viruses that expressed cell fusion-inducing glycoproteins such as the vesicular stomatitis virus (VSV) G glycoprotein (Pellinen *et al.*, 2004), or the gibbon ape leukemia virus envelope glycoprotein (Diaz *et al.*, 2000; Fu *et al.*, 2003). However, expression of these potent fusion glycoproteins may increase systemic toxicities due to glycoprotein leakage beyond the tumor boundaries. The gBSyn3 mutation causes virus-induced cell fusion limited to the site of infection eliminating the chance for fusion of cells at distal sites.

Treatment of Human Breast Cancer Mouse Xenografts

We reported previously that MM4L cells injected subcutaneously into nude mice establish tumors efficiently especially in the presence of Matrigel, which provides optimum scaffolding for tumor establishment (Fridman *et al.*, 1991; Mehta *et al.*, 1993; Bao *et al.*, 1994). Tumors reach relatively large sizes (approximately 70-80 mm^3) within three weeks post implantation and do not metastasize for at least five weeks post implantation. At much later times, MM4L cells are known to efficiently metastasize to lymph nodes and distal organs, including the lungs, liver, bones, gonads and brain (Leuschner, 2003; Leuschner *et al.*, 2006). The purpose of the present study was to determine the ability of the OncSyn virus to treat the implanted breast tumors within a time interval in which the tumor remained largely localized to the site of implantation. Many preclinical studies had indicated that oncolytic HSVs work best when administered in multiple doses (Carew *et al.*, 2001; Delman *et al.*, 2002). Preclinical data also indicate that HSVs can be delivered repeatedly and be effective in spite of cell-mediated and humoral responses (Breakefield and DeLuca, 1991; Delman *et al.*, 2002). Consistent with these reports, two rounds of three injections at different intratumor sites reduced tumor sizes by

approximately 80% as measured by manual measurements of tumor sizes. Quantification of chemiluminescence emitted by MM4L cells revealed more than one log-scale reduction of relative light intensities in comparison to the inactivated virus control injections. The discrepancy between the manual tumor size and the chemiluminescence measurments may to a large extent be due to the presence of localized cysts at the site of tumor establishment due to the presence of necrotic cells and remaining amounts of Matrigel. In this regard, the chemiluminescence signals may more accurately reflect the presence of viable MM4L cells within the tumors. This conclusion was supported by the pathological examination of the excised tumors, which revealed the presence of extensive necrosis of MM4L cells in virus-treated tumors. In contrast, pathological examination of both the inactivated virus and PBS control tumor samples, revealed the presence of viable MM4L cells unaffected by these treatments.

The NV1020 virus has been recently shown to be safe in human trials in a recent Phase I, open-label, dose-escalating study for subjects with metastatic colorectal carcinoma to the liver (Kemeny *et al.*, 2006), as well as efficacious in combination with chemotherapy (Gutermann *et al.*, 2006). One of the potential significant advantages of the NV1020 over the G207 virus is that it may require lower viral doses to achieve therapeutic effects. Furthermore, we hypothesize that the OncSyn virus may be delivered in lower viral doses than the NV1020 virus due to its potential advantage in virus production and intratumor spread over the NV1020 virus. A direct comparison of the in vitro and in vivo replication characteristics of NV1020, G207 and OncSyn viruses should clarify this issue. In this study, in vivo experiments in mice have revealed that the OncSyn virus is heavily attenuated in both nude and immunocompetent mice. However, additional safety data need to be obtained before the virus can be tested in human subjects. The OncSyn virus was re-isolated as a bacterial artificial chromosome (pOncSyn), which will enable the rapid construction of additional viruses that may exhibit increased efficacies in

in breast cancer treatments. Potential future modifications may include placing the remaining γ_1 34.5 gene under a breast tumor-specific promoter control, as well as include one or more transgenes that can enhance the oncolytic properties of the virus. In this regard, the large deletion of the virus renders the incorporation of multiple gene cassettes feasible without being limited by viral genome packaging restrictions.

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CHAPTER III

NV1020-LIKE HERPES SIMPLEX VIRUS TYPE-1 ONCOLYTIC AND HIGHLY FUSOGENIC MUTANTS EFFECTIVELY INHIBIT PRIMARY AND METASTATIC TUMORS IN MICE

Introduction

Recent advances in molecular virology have enabled investigators to construct viruses that selectively destroy cancer cells (oncolytic virotherapy). Genetically engineered viruses belonging to different viral families have been evaluated for their potential as therapeutic agents in the treatment of malignant tumors (Chiocca, 2002; Nemunaitis, 2002; Nemunaitis and Edelman, 2002; Kirn, 2003). Efficient replication, cell lysis and spread of HSV, and their natural broad host range make them attractive candidates as oncolytic viral agents (Lachmann, 2004; Fu *et al.*, 2006; Fu *et al.*, 2007). Furthermore, the recent availability of cloned HSV genomes into bacterial artificial chromosome vectors greatly facilitates the rapid construction of new recombinant viruses carrying multiple transgenes of interest (Meseda *et al.*, 2004; Jeyaretna and Kuroda, 2007; Schmeisser and Weir, 2007). Tumor treatment with oncolytic HSV has been shown to induce anti-tumor immune responses (Todo *et al.*, 1999; Miller and Fraser, 2003; Thomas and Fraser, 2003; Nakamori *et al.*, 2004; Li *et al.*, 2007). Although the majority of people are seropositive for HSV-1, oncolytic virotherapy with HSV is not limited by pre-existing anti-HSV immunity (Kemeny *et al.*, 2006; Hoffmann *et al.*, 2007), and in at least one example, preexisting immunity to HSV-1 enhanced anti-tumor immune responses (Miller and Fraser, 2000).

Recently, the NV1020 oncolytic herpes simplex virus type-1(HSV-1) was shown to have significant promise for the treatment of many different types of tumors in preclinical studies in experimental animals as well as in human clinical trials (McAuliffe *et al.*, 2000; Cozzi *et al.*, 2001; Cozzi *et al.*, 2002; Gutermann *et al.*, 2006; Kemeny *et al.*, 2006). The main advantage of

this virus over other HSV oncolytic viruses is that it expresses one of the two original $\gamma_1$34.5 genes allowing the virus to replicate more efficiently, while safety is not compromised (Meignier *et al.*, 1988; Meignier *et al.*, 1990; Cadoz *et al.*, 1992; Advani *et al.*, 1999). The $\gamma_1$34.5 gene is a major neurovirulence gene and an inhibitor of cellular apoptosis. Deletion of this gene drastically attenuates the virus and restricts viral growth to cancer cells because of their lack of intact apoptotic mechanisms (Chou *et al.*, 1990; Chou and Roizman, 1992). Preclinical studies in mice as well as phase I/II human trials have revealed that oncolytic HSV-1 viruses having both $\gamma_1$34.5 genes deleted did not spread efficiently within tumors (Kramm *et al.*, 1997). In contrast, deletion of one of the two $\gamma_1$34.5 genes drastically attenuated the virus, while allowing improved virus replication and spread in tumor cells (Meignier *et al.*, 1988; Meignier *et al.*, 1990; Advani *et al.*, 1999). The NV1020 was originally constructed for vaccine purposes and it contains HSV-2 viral sequences coding for glycoproteins gD, gG, gI and gE to facilitate production of anti-HSV-2 immune responses (Meignier *et al.*, 1988).

HSV can be transmitted from cell-to-cell by causing limited amounts of virus-induced cell fusion, thus avoiding the extracellular environment. Specific mutations within viral glycoproteins are known to greatly enhance virus-induced cell fusion. Specifically, syncytial mutations that cause extensive virus-induced cell fusion can arise in at least two of the glycoprotein genes: the UL27 gene, encoding glycoprotein B (gB) (Bzik *et al.*, 1984b, a; Pellett *et al.*, 1985), and the UL53 gene, coding for glycoprotein K (gK) (Bond and Person, 1984; Debroy *et al.*, 1985). Work in our laboratory has shown that gK functions as a heterodimer with the UL20 viral protein and the UL20/gK heterodimer is necessary for virus-induced cell fusion (Foster *et al.*, 2004; Foster *et al.*, 2008).

The HSV-1 oncolytic virus Onc was constructed based on the NV1020 genomic arrangement with the exception that there are no genomic re-arrangements and no HSV-2 genes

inserted within the viral genome. Recently, we reported that the OncSyn virus carrying a syncytial mutation in gB, enabling the virus to spread among cells by virus-induced cell fusion, replicated efficiently in breast cancer cells *in vitro* and drastically reduced tumor volumes *in vivo* (Chapter II, Israyelyan *et al.*, 2007). In this study we constructed and tested the OncdSyn virus, which in addition to the gBsyn3 mutation also carried the gKsyn1 mutation known to enable the virus to fuse even difficult to fuse cells (Manservigi *et al.*, 1977). Intra-tumor injections of either virus effectively reduced tumor volumes as well as inhibited tumor metastases to internal organs.

Materials and Methods

Cells

African green monkey kidney (Vero) cells and mouse mammary tumor cells (4T1) (Aslakson and Miller, 1992) were obtained from the American Type Culture Collection (Manassas, VA). Vero cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL; Grand Island, N.Y.), supplemented with 10% fetal calf serum (FCS) and antibiotics. 4T1 cells were maintained in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FCS. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Construction of the Doubly Fusogenic Recombinant Virus HSV-1 OncdSyn

The previously published OncSyn viral genome recovered as a bacterial artificial chromosome (bac) into *E. coli* (pOncSyn) (Israyelyan *et al.*, 2007) was used for the construction of pOncdSyn bac plasmid utilizing a new methodology – the double-red mutagenesis technique in *E. coli* (Tischer *et al.*, 2006) enabling the markerless introduction of the gKsyn1 mutation (Ala-toVal at aa 40). The OncdSyn virus was recovered after transfection of Vero cells with the pOncdSyn plasmid. The OncdSyn viral genome and the pOncdSyn bac were extensively characterized by diagnostic PCR and DNA sequencing to ensure the stability of the viral

genomes, the presence of the parental Onc deletions and the presence of the gKsyn1 mutation within the gK gene, as described previously for the OncSyn virus (Israyelyan *et al.*, 2007).

Phenotypic Characterization and Replication Kinetics of the OncSyn and OncdSyn Viruses

Cells (both Vero and 4T1) were seeded into 6-well plates and infected the following day (when they reached approximately 95% confluency) with the OncSyn or OncdSyn viruses at a multiplicity of infection (MOI) ranging from 0.001-1 plaque forming units per cell (PFU/cell). Cells were cultured in a maintenance medium (containing 2% FCS) and were left for 2 days to allow for the plaques and the cell fusion to develop. Photographs of the infected cells were taken using a fluorescence microscope. For assessment of viral plaque morphologies, Vero and 4T1 cells were infected with HSV-1(F), OncSyn or OncdSyn viruses and visualized after immunohistochemistry at 48 hours post-infection (h.p.i.) using horseradish peroxidase-conjugated anti-HSV antibody (Dako, Carpinteri, CA) and Novared substrate development kit (VectorLabs, Burlingame, CA).

To determine the replication kinetics of the viruses, one-step growth kinetics were performed as described previously (Foster *et al.*, 2001; Foster *et al.*, 2003). Briefly, nearly confluent monolayers of either Vero or 4T1 cells were infected with each virus at an MOI of 2 at 4°C for 1 h. Thereafter, virus was allowed to penetrate for 2 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment with phosphate buffered saline at pH 3.0. Cells and supernatants were harvested immediately thereafter (0 h) or after 12 or 24 h of incubation at 37°C. Virus titers were determined by endpoint titration of virus stocks on Vero cells.

Animal Experiments

Female Balb/c mice were obtained from Harlan (Indianapolis, IN) and housed in an animal room which was kept at 25°C with a 12 hour light-dark cycle. All experimental

procedures involving animals were approved by the institutional animal care and use committee (IACUC) of the Louisiana State University. At 6-7 weeks of age the animals (19-20g body weight) were implanted subcutaneously in the interscapular area with 1×10^5 viable 4T1 cells suspended in 0.2 ml of PBS using a 27 gauge needle. Body weights were determined weekly, and tumor sizes were monitored beginning 7 days after tumor inoculation by direct measuring with a digital microcaliper. Tumor volumes were calculated using the following formula: volume = (length × width × height)/2. At an average tumor volume of approximately 80-90 mm^3 (first experiment, day 13 post tumor cell inoculation) or 35-40 mm^3 (second experiment, day 9 post tumor cell inoculation), animals were randomized into 3 groups (first experiment) or 2 groups (second experiment) using a randomization plan (www.randomization.com). The groups of mice received 3 intratumoral injections of the OncSyn, OncdSyn viral particles, or PBS every four days for the first experiment and injections of the OncdSyn or PBS every third day for the second experiment. Each tumor was injected with approximately 1 x 10^6 viruses per injection in 250µl volume, while control mice received 250µl of PBS. Injections were performed slowly at 3 different sites per tumor. On day 42 for the first experiment and day 33 for the second experiment after initial tumor cell implantation, mice were humanely euthanized in a CO_2 chamber and subjected to gross as well as microscopic histological examination. Lung and other internal organ metastases were counted using a dissecting microscope after placing the resected organs in fixative for 24 hours. The primary tumor site, lungs, heart, liver, spleen, and kidneys from each animal were fixed in 10% neutral buffered formalin, trimmed, paraffin embedded, sectioned, stained with hematoxylin and eosin (H&E), and evaluated by light microscopy.

Statistical Methods and Analyses

The SAS® statistical package (Version 9.1.3) was used for the analyses of the *in vivo* studies. Distributions were examined for normality using the UNIVARIATE procedure with a

Shapiro-Wilk test of normality. For the repeated measures part of the analyses of tumor volumes and tumor weights, the GLM procedure was used to conduct a repeated measures design analyzed as a split-plot arrangement of treatments with TREATMENT (OncSyn, OncdSyn, and PBS) and MOUSE within TREATMENT as main plot factors. Subplot factors included PERIOD (days of measurements) and TREATMENT by PERIOD interaction. When overall analyses determined significance ($p \leq 0.05$), Tukey's HSD test was used to examine pairwise differences for main effects, and pairwise comparisons of least square means with regard to interaction effects were examined with preplanned t-tests. The Wilcoxon Two-Sample test was used to analyze the difference of lung metastatic node counts between PBS and OncdSyn groups.

Results

Construction and Characterization of the Oncolytic HSV-1 Mutant Virus OncdSyn

Previously, we described the construction and use of the NV1020-like virus OncSyn to treat human breast cancer utilizing a nude mouse xenograft model (Israyelyan *et al.*, 2007). To further increase the ability of the OncSyn virus to cause virus-induced cell fusion, the syncytial mutation gKsyn1 (Ala-to-Val at position 40) known to cause virus-induced cell fusion of even hard to fuse cells (Manservigi *et al.*, 1977) was introduced into the OncSyn viral genome cloned into a bacterial artificial chromosome (bac) using the markerless double-red mutagenesis method (Tischer *et al.*, 2006). The resultant OncdSyn virus carried syncytial mutations in both gB (syn3) and gK (syn1) (Figure 3.1). As we reported previously for the OncSyn virus, the bac-cloned OncdSyn viral genome was subjected to PCR-diagnostic analysis and direct sequencing of specific genomic loci to confirm the presence of the syn3 and syn1 mutations and the previously engineered deletion/insertion at the $\gamma_1$34.5 locus (not shown, Materials and Methods).

The plaque morphology of the HSV-1(F), OncSyn and OncdSyn viruses was examined on Vero cells and 4T1 cancer cells (Balb/c spontaneous mammary adenocarcinoma-derived)

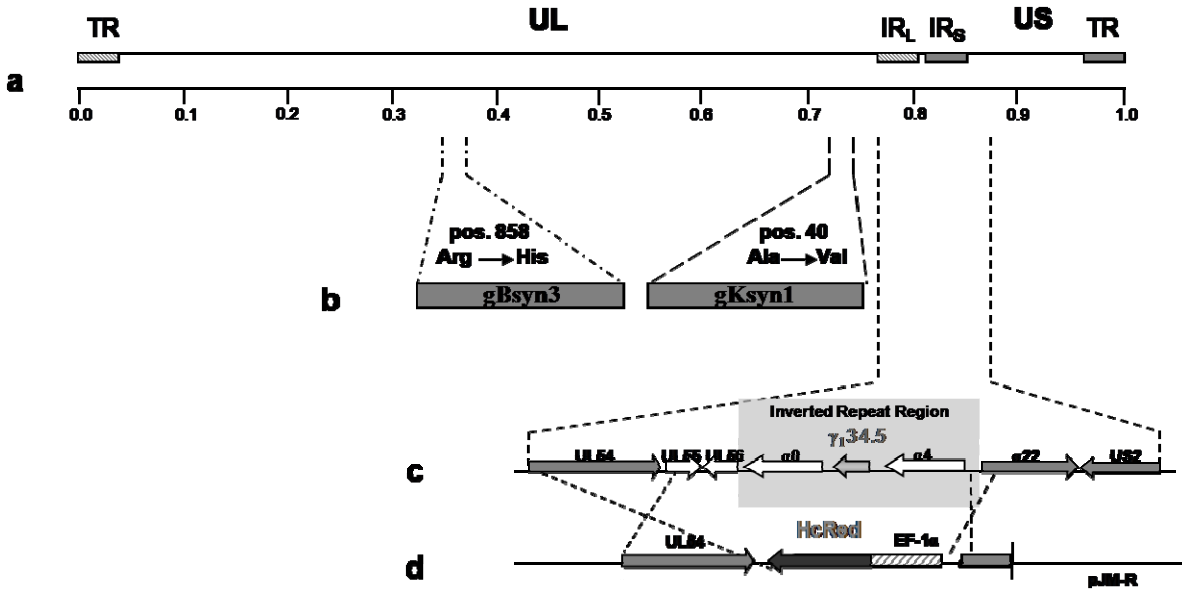

Figure 3.1. Schematic representation of the genomic structures of the oncolytic recombinant viruses OncSyn and OncdSyn. (a) Representation of the prototypic arrangement of the HSV-1 genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. (b) Approximate locations of the gB and gK genes. (c) An expansion of the inverted repeat region showing the approximate locations of UL54, UL55, UL56, α 0, $\gamma_1$34.5, α 4, α 22 and US2 genes. (d) Schematic of the DNA fragment cloned into plasmid pJM-R, which was used for insertion of the HcRed gene cassette into the viral genome in place of the NV1020 genomic deletion as described in Materials and Methods.

(Aslakson and Miller, 1992) as described in Materials and Methods (Figure 3.2). Plaque morphologies were visualized on Vero and 4T1cells at 48 hours post infection (hpi) by immunohistochemistry using a polyclonal anti-HSV-1 antibody (Figure 3.2: a-f). Mouse cells are known to be resistant to HSV-1 infection (Lopez, 1975; Kastrukoff *et al.*, 1986). Consequently, viral plaques generated by all three viruses tested were substantially smaller on 4T1 mouse cancer cells (Figure 3.2: d, e, f) in comparison to Vero cells (Figure 3.2: a, b, c). Specifically, the HSV-1(F) wild-type virus, which does not cause extensive virus-induced cell fusion, produced viral plaques on 4T1 cells that were approximately 10-fold smaller than those produced on Vero cells (Figure 3.2 d and a). In contrast, the OncSyn and OncdSyn viruses produced syncytial plaques on both cell lines tested (Figure 3.2: b, c, e, f); however, both the OncSyn and OncdSyn viral plaques on 4T1 cells were larger than those produced by the HSV-1(F) wild-type virus (Figure 3.2 e and f compared to d). The OncdSyn virus appeared to cause more pronounced virus-induced cell fusion on both Vero and 4T1 cells (Figure 3.2 c and f). In addition, the OncdSyn viral plaques emitted strong red fluorescence due to constitutive expression of the red fluorescence protein (RFP) expressed under the elongation factor 1α (EF-1α) promoter control (Figure 3.2 g and h), as it was previously reported for the OncSyn virus (Israyelyan *et al.*, 2007).

Kinetics of Viral Replication on Vero and 4T1 Cells

HSV-1(F) and OncSyn viruses replicated to similar titers in Vero cells, while the OncdSyn virus consistently replicated to titers that were a half-log lower than either HSV-1(F), or OncSyn viruses. The kinetics of viral replication were substantially slower in 4T1 cells than in Vero cells, and final titers in 4T1 cells were more than two logs lower for HSV-1(F) and OncSyn, while OncdSyn viral titers were more than three logs lower on 4T1 cells than in Vero cells. In addition, OncdSyn viral titers were approximately one log lower than the HSV-1(F) and OncSyn viral titers on 4T1 cells (Figure 3.3).

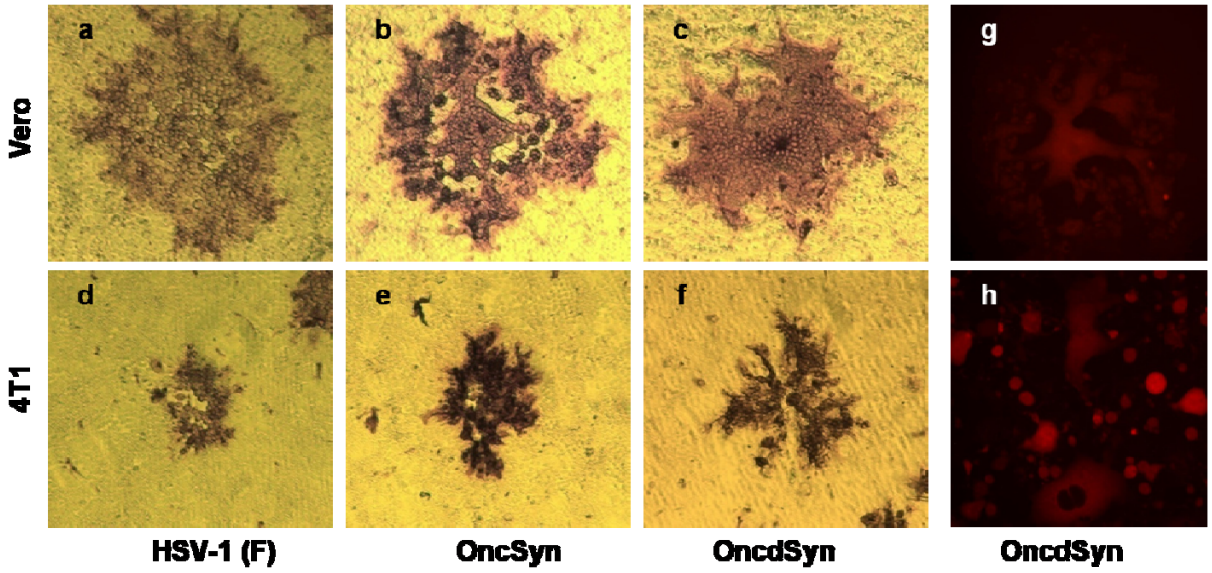

Figure 3.2. Plaque morphology of the HSV-1 (F), OncSyn and OncdSyn viruses. Nearly confluent Vero (a-c) and 4T1 (d-f) cell monolayers were infected with wild-type HSV-1(F) (a, d), OncSyn (b, e) and OncdSyn (c, f) viruses. Individual viral plaques were visualized 48 hr post infection by immunohistochemistry and photographed with a phase contrast microscope. Vero (g) and 4T1 (h) cells were infected with OncdSyn virus. Viral plaques were photographed 48 hr postinfection with a fluorescent microscope.

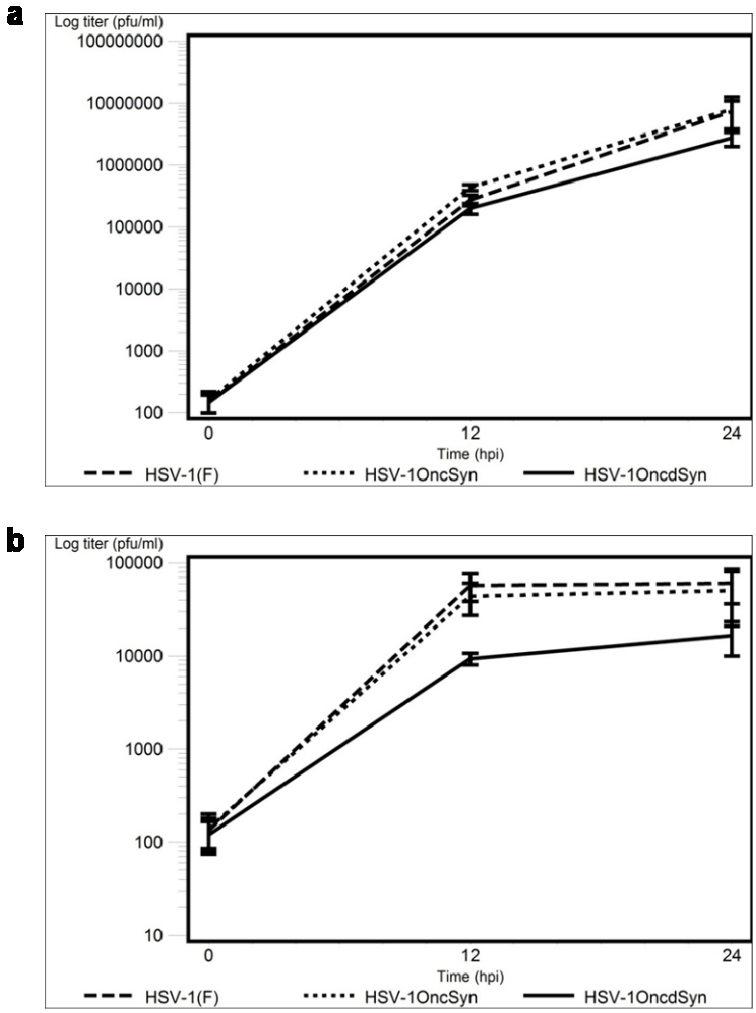

Figure 3.3. Comparative kinetics of viral replication of wild-type HSV-1(F) and mutant viruses OncSyn and OncdSyn grown on Vero and 4T1 cells. Near confluent monolayers of Vero (a) and 4T1 (b) cells were infected at an MOI of 2 with each virus, incubated at 37°C and the numbers of infectious virions were determined at different times post infection. Viral titers (mean pfu at each time point) are shown in logarithmic scale. The error bars represent means ± 2 standard errors.

Intra-tumor Virotherapy

4T1 cells were injected subcutaneously in the interscapular regions of Balb/c female mice. When the palpable tumors reached the volume of approximately 80-90mm^3, mice were injected with three consecutive intra-tumor injections of OncSyn and OncdSyn viruses or PBS (control) every four days as described in Materials and Methods. At the onset of viral intratumor injections, tumor sizes appeared similar in size for all three groups of mice ($p > 0.05$). Intratumor treatment with either OncSyn or OncdSyn virus caused a substantial reduction of tumor volumes in comparison to the PBS-treated control group of mice ($p < 0.05$). There was no significant difference in the reduction of tumor sizes in the two viral groups when compared to each other ($p > 0.05$) (Figure 3.4 a). Analysis of mouse weights during the course of the study did not show significant differences among the three groups, thus the efficacy of treatments was not affected by differential weight gain/loss in the groups (not shown) ($p = 0.296$). Representative tumors were excised immediately after mice were sacrificed. Typically, tumors treated with the PBS control injections appeared substantially larger than those treated with either the OncSyn or OncdSyn viruses (Figure 3.4 b).

The metastatic potential of the primary 4T1 tumor to internal organs after oncolytic or control therapy was assessed by gross and microscopic pathological examination of internal organs. In the first experimental protocol described above, mouse tumors were allowed to grow to approximately 80-90 mm^3 and mice were sacrificed at 42 days post tumor cell implantation. In this experiment, mouse lungs from all three groups of mice (PBS, OncSyn, OncdSyn) had numerous metastatic foci, which were too numerous to be accurately counted (not shown). However, tumor foci in liver and spleen were substantially reduced in OncSyn and OncdSyn-treated mice in comparison to PBS-treated control mice (Table 3.1, Figure 3.5). Specifically,

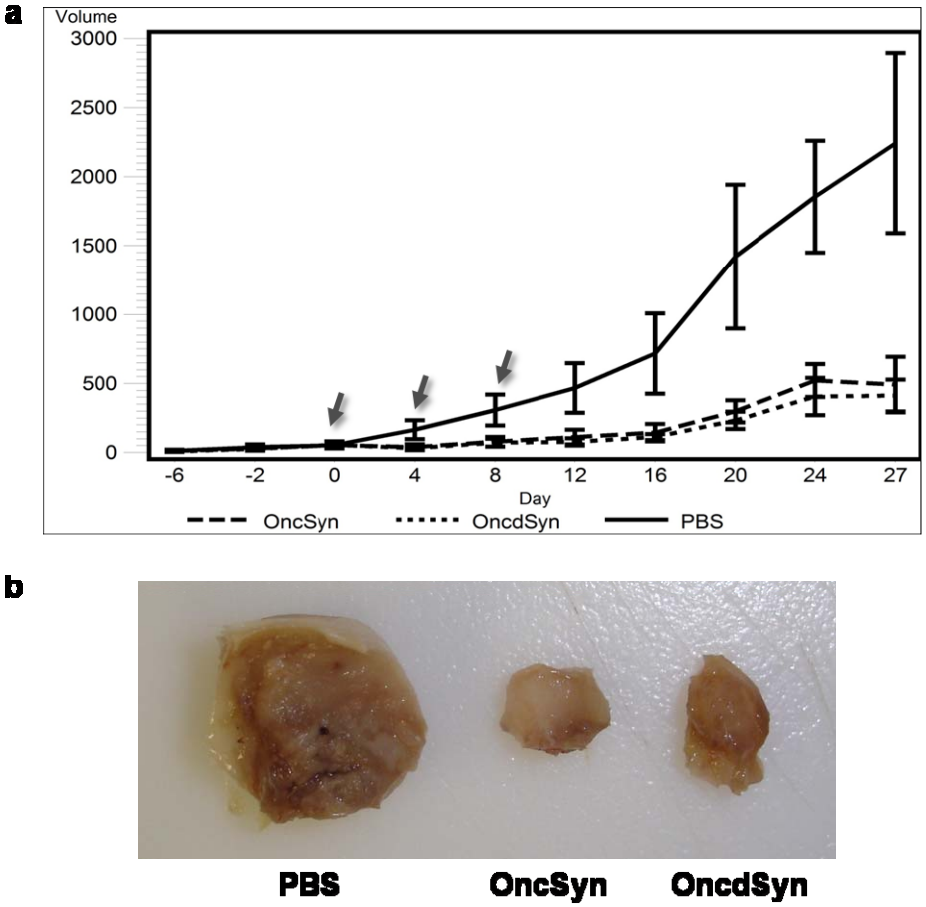

Figure 3.4. Intra-tumor treatment with OncSyn and OncdSyn viruses. (a) Balb/c mice were implanted subcutaneously in the interscapular area with 1×10^5 viable 4T1 cells. Tumors were measured using a digital caliper at defined time intervals prior and after treatment (x axis). Tumors were injected with either OncSyn, OncdSyn viruses, or PBS when tumors reached approximately 80-90 mm^3 in volume. Tumor volumes were measured prior to (negative values on the x axis) and after the injections. "0" on X axis represents the day of the first injection. The tumor volumes were determined from the formula: volume = (length × width × height)/2. Arrows indicate the days when therapy was administered. The error bars represent means ± 2 standard errors. (b) Tumors were excised at 42 days post implantation and visually examined. Panel shows representative tumors from virus and PBS treated animals.

all mice in the PBS group had metastatic nodes in liver, spleen, or kidneys. Some of the mice had tumors in all three organs (Figure 3.5). Importantly, there were no metastatic tumors observed in the kidneys of virus-treated mice (Table 3.1).

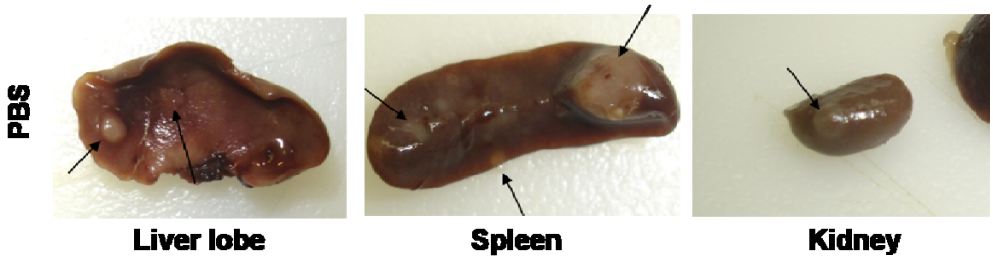

Figure 3.5. Gross pathological examination of metastatic tumor nodules on internal organs. Liver lobe, spleen, and kidney from a PBS-treated mouse carrying metastatic tumors (arrows) evaluated by gross pathological examination. Panel shows internal organs derived from a representative PBS-treated mouse.

Table 3.1: Metastatic nodes in internal organs

Experimental groups	No. of mice in group	No. of mice with metastases in internal organs	No. of mice with metastases in liver	No. of mice with metastases in spleen	No. of mice with metastases in kidney
PBS	**9**	9	6	7	3
OncSyn	**7**	4	2	2	0
OncdSyn	**7**	3	1	2	0

Experimental animals were sacrificed on day 42 post-injection of 4T1 cells and the internal organs were removed and examined for metastases formation by gross pathological evaluation as described in Materials and Methods.

To better assess the potential of oncolytic virotherapy to reduce metastatic tumors in internal organs, a second experiment was performed in a similar fashion to the previous one with the exception that in the new experiment tumors were allowed to grow to approximately 35-40 mm^3 in volume and mice were sacrificed at day 33 post tumor cell implantation after treatment with either OncdSyn or PBS. Lungs of OncdSyn-treated mice appeared to be practically devoid

of metastatic tumors with only two mice having two nodes each. In contrast, all PBS-treated mice had multiple metastatic tumors in their lungs (Table 3.2, Figure 3.6 a and b). These results were confirmed by pathological examination of paraffin-embedded lung sections stained with Hematoxylin & Eosin (H&E) staining, which revealed the absence of tumors in OncdSyn samples, while PBS-treated control samples had numerous visible tumor foci (Figure 3.6: c-f).

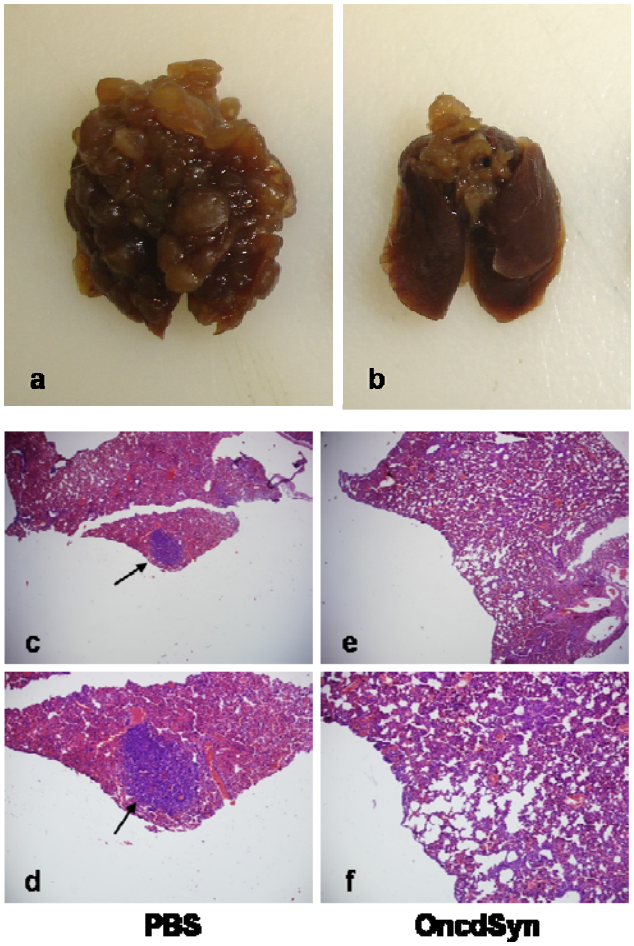

Figure 3.6. Therapeutic effect of OncdSyn virus on lung metastases. (a, b) Gross appearance of excised lungs of representative mice from PBS control and OncdSyn treated groups. (c-f) Lung tissues were stained with H&E and examined. Representative stained sections are shown for PBS (c, d) and OncdSyn (e, f) groups at 40X (c, e) and 100X (d, f) magnifications. Metastatic foci are represented by arrows (c, d).

Table 3.2: Metastatic nodes in lungs

Experimental groups	No. of mice in group	No. of metastatic nodes in lungs of experimental animals[a]							
		mouse1	mouse2	mouse3	mouse4	mouse5	mouse6	mouse7	mouse8
PBS	**7**	3	5	3	18	3	10	1	
OncdSyn	**8**	2	0	0	0	2	0	0	0

Experimental animals in PBS and OncdSyn groups were sacrificed on day 33 post-inoculation of 4T1 cells and the lungs were removed and examined for metastatic node formation as described in Materials and Methods. [a]PBS > OncdSyn (Wilcoxon Two-Sample test, $p = 0.002$).

Discussion

The oncolytic HSV-1-based virus NV1020 has shown strong promise for treatment of different tumors in animal models and human clinical trials (McAuliffe *et al.*, 2000; Cozzi *et al.*, 2001; Cozzi *et al.*, 2002; Gutermann *et al.*, 2006; Kemeny *et al.*, 2006). To facilitate the construction of recombinant viruses carrying multiple transgenes of interest, we cloned the NV1020-like HSV-1 recombinant virus OncSyn into a bacterial artificial chromosome (bac) vector. The OncSyn virus specifies a syncytial mutation in gB (Arg-to-His change at aa 858) that increases its ability to spread in tumor cells via virus-induced cell fusion (Israyelyan *et al.*, 2007). In this study, we introduced the syncytial mutation syn1 (Ala-to-Val change at aa 40) within the gK gene to further enhance the fusogenicity of the new virus OncdSyn. The OncdSyn virus reduced primary tumor sizes and inhibited metastases to distal organs in the 4T1 syngeneic mouse model system.

The syn1 mutation within gK has been shown to produce extensive virus-induced cell fusion in all cells tested. In comparison, the gBsyn3 mutation produced virus-induced cell fusion in most cells, but it was unable to fuse certain hard to fuse cells, such as Hep-2 cells (Manservigi *et al.*, 1977). Therefore, to further increase the ability of the OncSyn virus to fuse all types of cells, we generated the OncdSyn virus carrying both the gBsyn3 and gKsyn1 mutations. As expected, the OncdSyn virus caused extensive virus-induced cell fusion and fused Hep-2 cells,

while the OncSyn virus did not (not shown). Furthermore, the OncdSyn virus caused more extensive fusion than OncSyn in both Vero and 4T1 cells. The OncdSyn virus appeared to produce intact syncytia that remained attached to the cell culture flasks, while the OncSyn virus-induced syncytia contained infected single cells, which detached easier than the OncdSyn-infected syncytia. This phenomenon has been previously observed for the gB and gK syncytial mutations and it is probably due, in part, to the extensive virus-induced cell fusion caused by the gK syncytial mutation, which appears to also fuse internal membranes such as nuclear membranes in addition to plasma membranes of cells (Kousoulas, unpublished). Viral titers of the OncdSyn virus were lower in Vero cells than titers of the OncSyn virus and substantially lower than titers of the OncSyn virus in 4T1 cells. Typically, HSV-1 syncytial mutants produce lower viral titers than their parental wild-type viruses, most likely because of their direct effect on cellular membranes. In this regard, the increased ability of the OncdSyn virus to cause extensive virus-induced cell fusion is probably responsible for the observed decrease in viral titers in comparison to the OncSyn virus.

Defects of viral replication and spread in mouse cancer cells have been described in the literature for oncolytic herpesviruses (Thomas and Fraser, 2003; Nakamori *et al.*, 2004). HSV-1 does not replicate efficiently in mouse cell lines (Lopez, 1975; Kastrukoff *et al.*, 1986) most likely because it cannot as efficiently utilize the mouse nectin-1 receptor, which is approximately 5% different in its amino acid sequence to the human nectin-1 receptor (Shukla *et al.*, 2000). Nectin-1 is also known to facilitate virus-induced cell fusion and virus-spread (Even *et al.*, 2006). Consequently, both OncSyn and OncdSyn viruses replicated much less efficiently in 4T1 cells than in Vero cells. In this regard, the limited replication and spread of these viruses in 4T1 cells would be expected to adversely affect their oncolytic ability in 4T1-derived tumors *in vivo*. Previously, we reported that the OncSyn virus effectively reduced primary human breast cancer

tumors in nude mice (Israyelyan *et al.*, 2007). The disadvantage of the MDA-MB-435S human breast tumors is that these tumors would be rapidly eliminated if they were implanted in immunocompetent mice. Therefore, we chose the 4T1/Balb/c mouse model system for additional testing of both the previously constructed OncSyn virus as well as the newly constructed OncdSyn virus. Both OncSyn and OncdSyn viruses substantially reduced the growth of 4T1 tumors compared to the PBS controls, despite the fact that these viruses did not efficiently replicate in 4T1 cells in cell culture. Apparently, viral replication and infectious virus production in cell cultures did not correlate with the oncolytic efficacy of these viruses, because the OncdSyn virus reduced tumor volumes equally-well with the OncSyn virus, despite the fact that OncdSyn replicated approximately less than half a log than the OncSyn virus in 4T1 cells. Therefore, the relative increased ability of the OncdSyn virus to destroy tumors *in vivo* must be attributed to its enhanced fusogenicity.

Multiple murine tumor models have been used as preclinical settings for therapeutic purposes. The 4T1 mammary carcinoma model has several distinct advantages to be used as such model. It is regarded as a highly physiological, clinically-relevant mouse model that closely resembles stage IV human breast cancer in its properties (Aslakson and Miller, 1992). 4T1 cells are considered to be very weakly immunogenic (relative antigenic strength is less than 0.01 with 9.9 being the most immunogenic) (Reif, 1985; Miller, 1996), and they spontaneously metastasize to distal parts of the body (Aslakson and Miller, 1992; Pulaski and Ostrand-Rosenberg, 1998). Metastatic tumor foci in liver and spleen were substantially reduced in OncSyn and OncdSyn-treated mice in comparison to PBS-treated control mice. Reduction of metastatic foci in internal organs (lung, spleen, kidney and liver) was dependent on the size of the original 4T1 tumor, as well as the time of necropsy post implantation of tumor cells. Specifically, there was drastic reduction in tumor foci detected in lungs when the initial tumor size treated with the virus was

approximately 35-40 mm^3 and necropsies were performed at 33 days after tumor implantation. Furthermore, lungs appeared to have the same number of metastatic foci with PBS-treated controls when the initial treated tumors where 80-90 mm^3 and necropsies were performed at day 42 after tumor implantation. This metastatic pattern revealed that lungs were the primary metastatic site of the subcutaneous implanted 4T1 cells. Regardless of the size of the initial tumor treated and the time of necropsies post tumor implantation, it was evident that the tested viruses appeared to efficiently reduce the growth of the primary tumor as well as substantially inhibit or eliminate formation of metastatic foci.

It is highly likely that reduction of the primary tumor after oncolytic virotherapy with the OncSyn and OncdSyn viruses is responsible for the observed reduction in the formation of secondary tumor foci, since treatment of the smaller (35-40 mm^3) tumors appeared to drastically reduce lung metastases. Alternatively, it is possible that anti-tumor immune responses were elicited by exposure of tumor antigens after destruction of 4T1 cells within the primary tumor by the OncSyn and OncdSyn viruses. In this regard, a fusogenic oncolytic HSV-1 Synco-2D was reported to elicit anti-tumor immune responses when studied in a similar animal model of mammary carcinoma utilizing 4T1 cells (Nakamori *et al.*, 2004). A strong T-cell response was reported also by an HSV-2 derivative oncolytic virus FusOn-H2 effectively treating primary and metastatic mammary tumors *in vivo* (Li *et al.*, 2007).

Overall, our results showed that both OncSyn and OncdSyn viruses can efficiently reduce the primary and metastatic growth of 4T1 tumors established in immunocompetent mice. It is expected that these viruses would be even more efficacious against human breast cancer tumors by virtue of the fact that they can replicate substantially more efficiently (more than one log) in human than mouse cells. The availability of both OncSyn and OncdSyn viruses as bacterial artificial chromosomes will enable the generation of additional recombinant viruses that carry

multiple anti-tumor and immunomodulatory transgenes, which could further enhance the anti-tumor efficacy of these viruses.

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CHAPTER IV

CONCLUDING REMARKS AND FUTURE PROSPECTS

Currently, oncolytic virotherapy of cancers is an emerging biotherapeutic platform, which is largely based on the recent advances in the field of molecular biology and virology. Clinical data suggest that oncolytic viruses may offer substantial therapeutic advantages over existing cancer therapies such as chemotherapy and radiation. The three primary benefits identified to date include the following: (1) high therapeutic index; (2) better antitumor efficacy due to oncolytic replication; (3) synergistic antitumor activity with other cancer therapies. Oncolytic virotherapy may soon become the fifth modality of cancer therapy after surgery, radiation therapy, chemotherapy, and immunotherapy.

Over the past 15 years, multiple oncolytic recombinant mutant viruses have been engineered. HSV-1 was one of the first oncolytic viruses investigated for its therapeutic potential against cancer. Herpes simplex virus can be easily modified for oncolytic virotherapy. Among various oncolytic HSV-1 vectors reported recently, some carry transgenes, including immunomodulatory molecules, antiangiogenic peptides, prodrug-converting enzymes, etc. "Arming" HSV-1 with therapeutic transgenes is expected to greatly enhance its efficacy. Recently developed bacterial artificial chromosome-based systems enable the fast generation of recombinant HSV-1 vectors within weeks. During the tenure of this thesis work, our work focused on the generation of HSV-1 recombinant viruses cloned into bacterial artificial chromosomes and carrying specific mutations that enhanced the ability of these viruses to spread within tumors. Apparently, these viruses were efficacious in killing breast cancer cells in vitro and in animal mouse model systems. Thus, the major contribution of this thesis work is the development of two HSV-1 mutant viruses, which we hope will form the basis of additional virus mutants that could be ultimately used in human patients to treat a variety of tumors.

Of particular interest is the ability of HSV-1 oncolytic virotherapy to induce anti-tumor immune responses. Our in vivo experiments in immunocompetent mice revealed a substantial reduction of metastatic foci after intratumor therapy of primary transplanted tumors. Preliminary results to assess anti-tumor immunological responses were inconclusive. Additional studies are needed to further investigate the ability of these viruses to elicit anti-viral and anti-tumor immune responses. An exciting aspect of this work may be the expression of known cancer antigens via the HSV-1 vector to facilitate the dissection of antigen-specific immune responses.

HSV-1 oncolytic strains, such as the OncSyn and OncdSyn viruses can be easily modified to further increase their efficacy and safety. One drawback of both viruses is that although the inclusion of syncytial mutations allows for enhanced virus spread within the tumor, it also causes substantial reduction in viral replication and infectious virus production. This viral growth reduction combined with the reduction of infectious virus production due to lack of one of the two $\gamma_1$34.5 genes may render the production of high titer viral stocks for clinical use a difficult and expensive proposition. One potential way to overcome this virus replication defect is to engineer expression of the single $\gamma_1$34.5 gene under the promoter control of a gene, which is preferentially expressed in specific tumors targeted for therapy. In fact, this approach has been successfully tested for different promoters (Chung *et al.*, 1999; Hinoda *et al.*, 2003). Alternatively, $\gamma_1$34.5 can be provided from a cell line constitutively expressing this gene. Safety is another issue that needs to be addressed. In this regard, deletion of the ICP6 gene, which is known to be dispensible for virus replication in most cancer cells, but required for replication in normal cells, could be engineered to enhance the safety of the OncSyn and OndSyn viruses. Finally, deletion of the HSV-1 ICP47 gene, which is involved in MHC I downregulation, may be needed to enhance anti-viral and anti-tumor immune responses.

In most instances, oncolytic HSV mutant viruses have been delivered either within a tumor or proximal to a tumor. One could envision that viruses could be constructed to selectively bind and enter into specific cell types or tumors via taking advantage of the mechanisms that the virus utilizes for receptor binding and entry into cells. For example, it was recently shown that the HSV-1 gD glycoprotein, which is the main glycoprotein that binds the nectin-1 receptor in most cells, can be altered to change the binding and entry characteristics of the virus into different types of cells (Zhou *et al.*, 2002; Zhou and Roizman, 2007). Targeting of the virus using specific receptor-ligand interactions may ultimately allow systemic delivery of the virus as well as increased selectivity for specific tumors. A potential drawback of systemic HSV-1 delivery (intravenous) is pre-existing immunity against the virus, since most people are HSV-1 seropositive. However, sufficient HSV-1 virions may still be able to find specific tumors, if administered at sufficient high doses and proximal to the location of the tumors. In addition, it is possible, that administration of immunosuppressive drugs immediately prior to virotherapy could enhance systemic delivery of oncolytic viruses as has been reported recently (Ikeda *et al.*, 1999; Ikeda *et al.*, 2000).

In the real world, it is unfortunate that most exciting experimental results are often not translated to actual therapies and drugs for human use. The major hurdle to overcome is to convince the pharmaceutical industry that a product is not only highly efficacious, but even more importantly, that it can be produced in large amounts without compromising safety. First and foremost, one needs “clinical grade” product produced under current good manufacturing procedures (cGMP). This type of production requires the development of advanced process engineering methods usually performed by dedicated facilities within either biotechnology or pharmaceutical companies and academic centers. The cost for production of cGMP materials is usually on the order of $250,000-$1 million or more, even for a small clinical trial. Next, the

cGMP product must undergo formal pharmacological and toxicological testing. Typically, the product is tested in rodents as well as non-human primates. The cost of toxicology testing is usually in the $250,000-$500,000 range. The third step involves multiple regulatory approvals requiring lengthy reviews by local and national committees. Local approval usually includes a scientific review committee (SRC) as well as the institutional biosafety committee (IBC) and the institutional review board (IRB). Each of these committees typically find issues or concerns that need to be addressed, often prompting changes to the clinical trial. At the federal level, the National Institutes of Health Recombinant Advisory Committee (RAC) must review all protocols that involve gene delivery. In addition, an investigational new drug (IND) application must be filed with the Food and Drug Agency (FDA) in order to launch a clinical trial. The requirements by the FDA to prove safety in animal models prior to testing in humans are quite extensive and have been increasing over the past several years due to some adverse events in early clinical trials. One may argue that the bar should be lowered considerably when considering trials for patients who have exhausted all other options. For the typical new drug, these steps are usually performed by a pharmaceutical company. For specific therapeutic products with a reasonably high projected market value, companies are willing to incur these expenses because of the expectation of eventual large profits. In the absence of interest from relatively large pharmaceutical companies, however, these steps often represent major hurdles for investigators at academic centers. Thus the clinical translation of findings in the laboratory often takes years, and sometimes these hurdles prove too difficult to overcome even for the most determined investigator.

Despite the above mentioned problems, currently there are several ongoing trials sponsored by small biotechnology companies. The belief is that clinical trials with viral therapeutics are just beginning and these new treatments will play an important role in future

cancer therapies. There is still much to be learned and to be improved, but currently available data certainly indicate a highly promising perspective for HSV-1-based therapies. With active research in cancer biology, virology, immunology and other related fields, new therapies based on HSV-1 viral vectors are anticipated. Important issues currently under investigation focus on improving the efficacy and safety of these viruses, the inclusion, evaluation and selection of therapeutic transgenes, and the improvement of vector delivery efficiency and specificity. Further understanding of the biology of HSV-1 will improve our ability to manipulate it to our advantage. Some approaches aimed to vector optimization deserve attention: (1) using fresh clinical isolates of HSV-1, rather than the laboratory strains which may have accumulated undesired genetic alterations, as the backbone for genetic engineering (Liu *et al.*, 2003). Care must be exerted as these strains are conceivably more virulent, and thus posing a greater risk, than many of the lab strains. Alternatively, it is possible to isolate viral mutants with enhanced antitumor efficacy by serial passage of a laboratory strain in cancer cells under carefully designed conditions (Taneja *et al.*, 2001). Multiple doses of virus, combination therapies such as radiation, targeted viruses, and viruses that express foreign genes that augment antitumor responses are promising avenues of research with potential high clinical impact. Future efforts will focus on increasing the efficacy of viral delivery and spread, as well as developing imaging approaches to allow rational administration of the virus, including determining the proper timing and geographic location for injection into the tumor.

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ANNA F. ISRAYELYAN, JEFFREY M. MELANCON, LARRY G. LOMAX, INDER SEHGAL, CAROLA LEUSCHNER, MICHAEL T. KEARNEY, VLADIMIR N. CHOULJENKO, ABOLGHASEM BAGHIAN, and KONSTANTIN G. KOUSOULAS

Effective Treatment of Human Breast Tumor in a Mouse Xenograft Model with Herpes Simplex Virus Type 1 Specifying the NV1020 Genomic Deletion and the gBsyn3 Syncytial Mutation Enabling High Viral Replication and Spread in Breast Cancer Cells.

HUMAN GENE THERAPY 18:457–473 (May 2007)

I would greatly appreciate if you could please fax the letter of permission to the fax number below and/or mail it to the above address. Thank you in advance for your time and assistance.

Permission Granted
Karen Ballen 2/7/08

Baton Rouge, LA 70803 Tel: 225-578-9683 Fax: 225-578-9655

Ryan Menezes

TOTAL P.01

VITA

Anna Henrik Israyelyan was born in February, 1976, to Tagush Sargsyan and Henrik Israyelyan in Yerevan, Armenia. Two years later, Anna gained a brother and lifelong friend when Arman Israyelyan was born. Anna spent her childhood in Yerevan and received a secondary education while participating in many educational competitions (langauge, math, science, etc) at different administrative/municipal levels. She graduated with a medical degree from Yerevan State Medical University after Mkhitar Heratsi in 1999. The same year she started a specialization in epidemiology of infectious and noninfectious diseases in the Department of Epidemiology, National Institute of Health of Armenia. In August 2001, Anna enrolled in graduate school in the Department of Pathobiological Sciences at Louisiana State University. There she began the work on the early diagnostics of breast cancer by mRNA tumor markers in the laboratory of Dr. K. G. Kousoulas. She received her master's degree in 2003. After graduation, Anna continued her studies toward a doctoral degree in the same laboratory working under supervision of Dr K. G. Kousoulas on the treatment of breast cancer through genetically engineered novel oncolytic herpesviruses. Upon graduation, Anna will continue her training in the Baylor Institute of Immunology Research, Dallas, Texas, where she hopes to broaden her understanding of novel immonotherapeutic methods to treat breast cancer and make a beneficial contribution to the community through advanced medical research.