THE DEVELOPMENT OF HERPES SIMPLEX VIRUS VECTORS FOR CANCER AND GENE THERAPY

By

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B.Sc. (Microbiology and Immunology), University of British Columbia, 1996

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In

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(Deartment of Microbiology and Immunology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 2001

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ABSTRACT

The efficient delivery of therapeutic genes and appropriate gene expression are the crucial issues for clinically relevant gene therapy. Viruses are naturally evolved vehicles that efficiently transfer their genes into host cells. This ability made them desirable for engineering virus vector systems for the delivery of therapeutic genes. Among various vector systems, herpes simplex virus (HSV) vectors represent an attractive delivery system, since these vectors have high gene transfer efficiency and mediate high expression of therapeutic genes.

Although HSV has been shown to infect most cell types, they were restricted from mature skeletal muscle tissue. As a result, research involving the use of this vector for muscle-directed gene therapy was hampered. Previous studies indicated that the loss of infectivity may be due, at least in part, to the development of the basal lamina throughout the course of muscle maturation. Enzymatic disruptions of the basal lamina showed moderate increases in levels of infection, although marked toxicity with such procedures resulted. To initiate infection, HSV normally attaches to cell surface heparan sulfate, which stabilizes the virus such that it can interact with secondary protein receptors required for entry into host cells. Our studies revealed a downregulation of heparan sulfate biosynthesis during skeletal muscle maturation. Furthermore, infectivity could be restored by exposing mature skeletal myofibers to low concentrations of the glycosaminoglycan analog, dextran sulfate (DS). This molecule appears to act as a surrogate receptor to stabilize the virus at the myofiber surface such that HSV can engage additional receptors. This demonstration that the basal lamina is not an absolute block to HSV infection is remarkable because it allows for the nondestructive targeting of HSV to mature myofibers and greatly expands the usefulness of this vector for the treatment of inherited and acquired diseases.

In light of these results, dextran sulfate was further examined for its ability to target cancer cells in a systemic model of delivery. Cancer cells typically display altered glycosaminoglycan profiles, similar to mature skeletal muscle tissue. Vascular delivery of oncolytic HSV and DS significantly delayed tumor growth, with 25% of the animals cured following treatment. Although, DS did not act to stimulate infection of cancer
cells, its ability to alter the hemodynamic properties of the animal system in favor of viral accumulation at tumor portals was key. Surprisingly, viral replication was not necessary for antitumor efficacy and relatively low amounts of virus could result in marked oncolysis. Furthermore, immunohistochemistry revealed infection of tumor vasculature alongside very limited infection of surrounding tumor tissue. Taken together, the tumor vasculature is likely the major target for oncolytic HSV in a systemic delivery model of cancer. Thereby efforts to further enhance delivery of oncolytic HSV to tumor vasculature by incorporating targeting peptides and using antiangiogenic viruses have been successful.

Understanding the biology of gene therapy systems is crucial to developing the most efficient and specific systems suited for individual disease applications. Increased insights into the entry and trafficking of gene therapy systems in animal models facilitates two approaches to developing appropriate therapies for individual applications. First, understanding more clearly the biology of currently available systems permits a more judicious choice of applications. Second, this also forms the basis for development of advanced delivery systems with increased efficiency, stability and targeting specificity. Therefore studies that provide insight into why biological therapies succeed and fail not only allow for a better understanding of animal and vector systems, but they allow us to exploit this knowledge to improve our arsenal of standard protocols of care for disease.
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<td>adeno-associated virus</td>
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<tr>
<td>ACV</td>
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<td>ALV</td>
<td>avian leucosis virus</td>
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<tr>
<td>AIDS</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BSA</td>
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PREFACE

The work presented in this thesis denotes research efforts by the candidate from 1997 to 2001. Below is the list of papers that have been published as a result of this work, and the contributions made by the candidate:


The candidate is responsible for all of the work presented in this study. K. Bockhold taught the candidate to do primary myofiber cultures.


The candidate is responsible for the writing of this review.


The candidate is responsible for the writing of this chapter.


The candidate is responsible for all of the work in this study. Animal experiments were conducted by the candidate and a team of scientists (D. Qiang, K.A. Delman, J.S. Zager).

• **S.N. Yeung** was also invited by the editor of *Current Gene Therapy* to submit a review on viral targeting for tissue-specific gene therapy. This review is currently being written by the candidate.
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CHAPTER 1: Introduction

1.1 Overview

Viruses are obligate intracellular parasites that are unable to replicate on their own. Therefore they must enter a host cell and scavenge the host cell energy supplies and biosynthetic machinery to serve their own replication. Because of their dependence on the host cell, they must use the signals and regulatory pathways of the host. In this manner, the study of viruses has consistently led to a greater understanding of the host cell biology. Many of the concepts and tools of molecular biology have been derived from the study of viruses and their host cells. Through the study of retroviruses and DNA tumor viruses, we also owe a great proportion of our present understanding of human cancers to the field of virology. The medical consequences of viral infections of humans have resulted in extraordinary efforts to study, understand, and eradicate these pathogens. Therefore, the study of viruses has extended to vaccine development and significant advances in epidemiology. Furthermore, the relationship between host and virus has been extensively investigated for the development of viral vectors for the treatment of human disease. In recent years, many diseases have been characterized on a molecular level. The knowledge of the pathophysiological alterations is the first step toward the development of new treatment strategies based on gene therapy. Therefore as we learn more about how viruses enter and pirate target hosts, there will be significant advances to engineering viral vehicles to deliver therapeutic genes and to kill tumor cells.

To gain entry into the host cell, viruses use host cell surface receptors that normally serve as receptors for other molecules. HSV uses heparan sulfate (HS) glycosaminoglycans/(GAGs) as receptors for initial attachment to the host cell surface. This interaction tethers the virus to the cell surface such that it can interact with secondary protein receptors for entry, and consequently productive infection. Cells devoid of HS are typically resistant to viral infection. However, GAG analogs such as DS have been used as artificial receptors to mediate HSV infection of resistant cell lines. In this light, I applied this property of DS to expand the usefulness of HSV vectors for muscle-directed gene therapy. Prior to this work, HSV was not amenable to gene therapy
for muscle disease because skeletal muscle is resistant to HSV infection. Along these lines, the use of skeletal muscle as a neo-organ for the production of therapeutic proteins was also discounted because HSV vectors carrying such genes could not gain entry into skeletal muscle tissue. As a consequence to successfully overcoming this block to HSV infection by the use of DS, the platform for use of HSV in muscle-directed gene therapy has been greatly expanded.

A similar approach was used to make HSV suitable for systemic treatment of cancer. Systemic delivery has the advantages of tailored delivery for microscopic and metastatic disease. In this case, the hemodynamic properties of DS were exploited to target oncolytic HSV to tumor. This study has not only led to the development of an effective treatment of colorectal cancers, but it has enabled the unraveling of a working model of how systemic delivery of HSV works in animal models of cancer. The importance of this lies in our ability to exploit this knowledge to make future therapies safer and more effective.

The following introduction addresses the structure and properties of GAGs and GAG analogs. I also review the process of HSV infection and the clinical implications of this virus for human disease. As DS plays a central role in improving the use of HSV for cancer and gene therapy, I will expand on its molecular uses to include its clinical properties and benefits. In addition, the advantages of choosing HSV as a vector will be reviewed, highlighting muscle systems and cancer models as specific diseases of interest. This will set the stage for a discussion of the implications of this research on the treatment of both genetic and acquired diseases, as well as the future benefits of molecular therapies in medicine.

1.2 Glycosaminoglycans and Proteoglycans

Large numbers of proteins in animal tissues occur immobilized in the extracellular space, at cell surfaces or in the extracellular matrix. Some are anchored through interactions with other proteins. However, current research increasingly implicates proteoglycans as scaffold structures, designed to accommodate proteins through noncovalent binding to their GAG side chains (Kjellen and Lindahl 1991).
In particular, HS proteoglycans are recognized as ubiquitous protein ligands. Binding of proteins to HS chains may serve a variety of functional purposes, from simple immobilization or protection against proteolytic degradation to distinct modulation of biological activity (Figure 1.1). Because of such interactions, HS proteoglycans are critically involved in a variety of biological phenomena at various levels of complexity, including organogenesis in embryonic development, angiogenesis, regulation of blood coagulation and growth factor/cytokine action, cell adhesion, and lipid metabolism (Lindahl, Lidholt et al. 1994; Salmivirta, Lindholt et al. 1996; Rosenberg, Shworak et al. 1997).

1.2.1 Glycosaminoglycans

A GAG is a linear heteropolysaccharide consisting of specific repeating disaccharide units (Jackson, Busch et al. 1991). Usually one monosaccharide of the disaccharide repeats is a hexuronic acid, either glucuronic acid (GlcA) or iduronic acid (IdoA), and the other sugar is a hexosamine, either N-acetylg glucosamine (GlcNAc) or N-acetylgalactosamine (GalNac). One or both of the sugars are variably N- and O-sulfated, which contributes to the diversity of these structures and their high negative charge density. The most common GAGs include heparin, HS, dermatan sulfate and chondroitin sulfate (CS) (Table 1.1). These can be linked through an O-glycosidic linkage to a serine residue in a protein core. A GAG covalently linked to a protein in this manner is termed a proteoglycan. Proteoglycans and their attached GAGs have a variety of roles in cell-cell interactions and serve as activators of growth and anti-coagulation factors.
Figure 1. An example of the role that heparan sulfate proteoglycans can play in facilitating receptor-ligand interactions at the cell surface.

In the figure, fibroblast growth factor-2 (FGF2, mauve triangles) is only recognized by FGF receptors after it has been activated (mauve circles) by interaction with heparan sulfate chains (Spivak-Kroizman, Lemmon et al. 1994). Ligand binding to FGF2 receptors results in a ternary complex, and these activated FGF receptors then form dimers initiating a signal transduction cascade via the cytoplasmic C-termini. Both syndecans and the glycosylphosphatidylinositol (GPI)-linked glypicans are actively shed from the cell surface, which might facilitate the diffusion of growth factors and other molecules between cells (Edgren, Havsmark et al. 1997; Liu, Casey et al. 1998).
Table 1.1 Occurrence and properties of glycosaminoglycans.

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>MW (x10^3)</th>
<th>Repeating period monosaccharides</th>
<th>Sulfate per disaccharide unit</th>
<th>Examples of occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>4 – 8000</td>
<td>D-glucuronic acid D-glucosamine</td>
<td>0</td>
<td>connective tissue, skin, synovial fluid, umbilical cord cartilage, vitreous humour</td>
</tr>
<tr>
<td>Chondroitin 4- and 6-sulfates</td>
<td>5 – 50</td>
<td>D-glucuronic acid D-galactosamine</td>
<td>0.1 – 1.3</td>
<td>cartilage, cornea, bone, skin, arterial wall</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>15 – 40</td>
<td>L-iduronic acid D-galactosamine</td>
<td>1.0 – 3</td>
<td>skin, heart valve, tendon, arterial wall</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>30^a</td>
<td>D-glucuronic acid or L-iduronic acid D-glucosamine</td>
<td>0.4 – 2</td>
<td>lung, arterial wall, ubiquitous on most cell surfaces</td>
</tr>
<tr>
<td>Heparin</td>
<td>6 – 25</td>
<td>D-glucuronic acid or L-iduronic acid D-glucosamine</td>
<td>1.6 – 3</td>
<td>lung, liver, skin, intestinal mucosa (mast cells)</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>4 – 19</td>
<td>D-galactose D-glucosamine</td>
<td>0.9 – 1.8</td>
<td>Cartilage, cornea, invertebral disc</td>
</tr>
</tbody>
</table>

Table reproduced from Lindahl and Hook, 1978 (Lindahl and Hook 1978).

^aAverage MW of HS chain (Sanderson, Huckerby et al. 1989).
1.2.1.1 GAG structure

Heparan sulfate, which is produced in nearly all cell types studied, and heparin, which is synthesized by connective tissue-type mast cells, are both synthesized as proteoglycans (Lind, Lindahl et al. 1993). Biosynthesis of HS and heparin proteoglycans involves the initial formation of a GAG structure composed of alternating GlcA and GlcNAc units (Figure 1.2). This initial structure is modified through a series of reactions, which leads to the formation of polymers with extensive structural diversity. By comparison, the modifications of CS are much less extensive, resulting in a relatively homogeneous molecule. CS is synthesized from an initial polymerization product consisting of alternating GlcA and GalNAc (Figure 1.2). Ester-linked sulfate groups are added typically at the C-4 and/or C6 positions on GalNAc residues. In some instances, the GlcA residue epimerizes to IdoA. In this case, the polysaccharide is called dermatan sulfate or chondroitin sulfate B.
Figure 1. HS and CS biosynthesis.

Abbreviations are as follows: Ser (serine), Xyl (xylose), Gal (galactose), GlcA (glucuronic acid), GlcNAc (N-acetylglucosamine), GalNAc (N-acetylgalactosamine).
1.2.1.2 Chain initiation

Synthesis of CS and HS begins after translation of the core protein and transfer of xylose to specific serine residues in the core protein (Kjellen and Lindahl 1991). Xylosylation is catalyzed by xylosyltransferase using UDP-xylose as the sugar donor and there is some evidence that this begins in the ER and continues in the Golgi (Silbert and Sugumaran 1995). In several proteins, xylosylation occurs on Ser-Gly-X-Gly (S-G-X-G) tetrapeptide sequences (where X is variable). The existence of a consensus sequence for GAG attachment is, however, controversial. The formation of polysaccharide chains continues in the cis and medial Golgi by the addition of galactose (Gal) and GlcA residues. This results in the formation of what is termed the tetrasaccharide linkage region (-glucuronic acid-galactose-galactose-xylose-serine), which is common to CS, heparin and HS proteoglycans. The pathway diverges after the formation of the linkage tetrasaccharide. At this point, the addition of GalNAc or GlcNAc commits the structure to be synthesized as either CS or HS/heparin, respectively. Kitagawa and colleagues (1999) recently demonstrated that the gene EXTL2 encodes an enzyme, which transfers GalNAc and GlcNAc to the common GAG-protein linkage region (Kitagawa, Shimakawa et al. 1999). Thus EXTL2 is likely the critical enzyme that determines whether the polysaccharide chain is to be HS or CS.

1.2.1.3 Biosynthesis of heparin and HS

Polymerization of GAGs begins after the formation of the tetrasaccharide linkage (Silbert and Sugumaran 1995). In the case of HS, polymerization is catalyzed by heparan sulfate polymerases, including those encoded by EXT1 and EXT2 (Lind, Tufaro et al. 1998). The GAG chain is formed by the transfer of alternating GlcA and GlcNAc monosaccharides from UDP-sugar nucleotides. Coupled with polymerization is the first modification of the GlcA-GlcNAc polymer, N-deacetylation, with subsequent N-sulfation of the GlcNAc units (Salmivirta, Lindholt et al. 1996). This is followed by C-5 epimerization of GlcA units to IdoA, which is coupled with O-sulfation at C-2. Finally, GlcNSO$_3$ units are modified by O-sulfation at C-6. The stepwise nature of this process should be emphasized, as the product of any given reaction will be the substrate for the next series of modifications (Salmivirta, Lindholt et al. 1996).
1.2.2 Proteoglycans

Proteoglycans (PG) are complex macromolecules consisting of a protein core to which are covalently attached one or more sulfated GAGs. PGs are ubiquitous components of cell surfaces and the extracellular matrix, and their GAG chains (which are highly negatively charged and occupy a relatively large amount of space) contribute to many biological functions (for a comprehensive review of proteoglycans, see (Kjellen and Lindahl 1991)). The localization of proteoglycans to the plasma membrane and extracellular matrix makes them important intermediates between cells and their environment. Some of these include modulation of enzyme activities, regulation of cell growth, and control of assembly of the extracellular matrix (Jackson, Busch et al. 1991). More specifically, they have been implicated to play a role in cell-cell (Dietrich, Sampaio et al. 1977) and cell-matrix (LeBaron, Hook et al. 1989) interactions, organization of basement membranes (Kleinman, McGarvey et al. 1983), control of macromolecular diffusion (Kanwar, Linker et al. 1980), and also interactions with a variety of ligands such as growth factors, hormones, and neurotransmitters (Kjellen and Lindahl 1991).

1.2.2.1 Core proteins

Many distinct proteins serve as core proteins for proteoglycans. It is the combination of a core protein and specific GAG chains that bestows a unique structure and function on any given proteoglycan. There are three general classes of proteoglycans. The first class includes proteoglycans that are secreted and deposited into the extracellular matrix (Esko 1991), such as aggrecan and basement membrane proteoglycans. The second family comprises PGs that are anchored to the plasma membrane, such as syndecan. PGs are associated with the plasma membrane in three different ways; intercalation of the core protein into the plasma membrane (Marynen, Zhang et al. 1989; Saunders, Jalkanen et al. 1989), intercalation through a glycosylphosphatidylinositol (GPI) anchor covalently attached to the core protein (Ishihara, Fedarko et al. 1987; Yanagishita and McQuillan 1989; Carey and Stahl 1990; David, Lories et al. 1990; Brunner, Gabri love et al. 1991), or interactions between the GAG side chains and other molecules on the cell surface. The third group of PGs comprises those that are typically found intracellularly in secretory granules and includes...
the PG serglycin. Some core proteins contain only one or few GAG chains, while others can contain as many as 100 or more. Moreover, core proteins can contain more than one type of GAG. Treatment of BHK cells with phosphatidylinositol (PI)-specific phospholipase C partially reduced HSV-1 binding to cells (Langeland and Moore 1990). This suggests that PI-linked proteoglycans are among the PGs that serve as receptors for the virus. Further studies are required to identify specific proteoglycans mediating viral attachment.

1.3 Viral Entry and Tissue Tropism

The initial interaction of a virus with its host cell is a key determinant of host range and tissue tropism. Broad host range viruses bind to widely distributed cell surface molecules. Influenza virus, for example, attaches to sialic acid moieties (Rossman 1994). Narrow host range viruses, on the other hand, recognize very specific molecules, as exemplified by rhinoviruses, which bind to intercellular adhesion molecule-1 (ICAM-1). Structural features of viral envelope glycoproteins are also key determinants of viral tropism. The host range of avian leucosis virus (ALV), for example, is defined by the env gene, which encodes two glycoproteins (Bova, Manfredi et al. 1986). Nucleotide differences in the env genes of different ALV subgroups permit the virus to use different host cell receptors. Similarly, the conformation of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein has been shown to play a role in the host range of this virus (Stamatatos and Cheng-Mayer 1993). Cellular proteinases, which can activate particular viral fusion proteins involved in penetration, also play a pivotal role in tissue tropism (Nagai 1995). The availability of a particular proteinase in the host, as well as the cleavability of the viral fusion protein, can determine the tropism of some viruses, such as influenza A. As discussed in more detail later, HSV interacts with specific combinations of receptors for entry into host cells. A key determinant of tissue tropism for HSV lies with the display of receptors by the host cell.

1.3.1 Glycosaminoglycan Interactions with Proteins

For many proteoglycans, proper functioning requires the interaction of the core protein with extracellular or intracellular ligands, along with interactions between GAG side chains and extracellular ligands. Binding of proteins to GAGs is generally
electrostatic in nature, although other types of interactions may occur. The GAG binding regions in proteins are usually clusters of basic amino acids. These GAG binding regions often have a preference to interact with the more highly sulfated, and therefore highly negatively-charged regions on GAGs. Frequently, binding can be of low specificity and affinity (electrostatic binding). In this instance, different polysaccharide sequences would be able to interact with a given protein. Conversely, binding can be of high specificity and affinity. For example, antithrombin binds with high affinity to a specific pentasaccharide sequence (Salmivirta, Lindholt et al. 1996). Thus the arrangement of sulfate groups and carboxyl groups, as well as the sugar composition of the polysaccharide can also influence protein binding. The best-described examples of this specificity are the pentasaccharide sequences recognized by antithrombin and fibroblast growth factor-2 (FGF2) (Lindahl, Lidholt et al. 1994).

1.4 Pathogens That Use HS for Entry

The structural complexity of GAGs, as well as their abundance on the surface of almost all mammalian cells, has made them attractive targets for a number of important pathogens. These pathogens include bacteria, like Chlamydia trachomatis and Neisseria gonorrhoeae as well as protozoans like Leishmania (Rostand and Esko, 1997). HSV was the first virus shown to bind HS (WuDunn and Spear 1989). Since then, other herpesviruses have been found to use HS as an initial receptor, including human cytomegalovirus (HCMV) (Compton, Nowlin et al. 1993), pseudorabies virus (PrV) (Mettenleiter, Zask et al. 1990), and bovine herpesvirus type 1 (BHV-1) (Okazaki, mitsuzaki et al. 1991). Recent reports have also implicated HS as a receptor for HIV (Patel, Yanagishita et al. 1993), human herpesvirus 7 (HHV-7) (Secchiero, Sun et al. 1997), type O foot and mouth disease virus (Jackson, Ellard et al. 1996), respiratory syncytial virus (Krusat and Streckert 1997), Dengue virus (Chen, Maguire et al. 1997), Sindbis virus (Klimstra, Ryman et al. 1998), vaccinia virus (Chung, Hsiao et al. 1998), porcine reproductive and respiratory syndrome virus (Jusa, Inaba et al. 1997), equine arteritis virus (Asagoe, Inaba et al. 1997), and adeno-associated virus (AAV) (Summerford and Samulski 1998). Studies of the nature of the interactions between those microbes and the cell surface GAGs reveal that most of the microbe binding
activity consists of reversible, low-affinity interactions. Thereby the microbe requires subsequent high-affinity interactions with specific cell surface receptors to complete the process of entry into the host cell.

1.5 The Herpesviruses

The family *Herpesviridae* comprises a group of large, enveloped double-stranded DNA (dsDNA) viruses that infect a broad host range of tissues from an equally broad range of species. The herpesvirus family consists of three subfamilies, the alpha-, beta-, and gamma- herpesvirinae, which share common features of size, genomic homology, and structure (Roizman and Sears, 1996). *Alphaherpesvirinae*, which include herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), PrV, and BHV-1, are characterized by a relatively short, 24-hour replication cycle, their variable host range, and rapid development of cytopathic effect (CPE) in cell culture. They also have a preference for epithelial and neural tissues, and can establish latency in sensory ganglia. *Betaherpesvirinae* consist of HCMV, human herpesvirus-6 (HHV-6) and HHV-7, and typically have longer replication cycles. Infected cells often become enlarged (cytomegalia) both *in vivo* and *in vitro*. The *gammaherpesvirinae* are lymphotrophic, targeting B and T lymphocytes, and are represented by Epstein-Barr virus (EBV) and the recently identified Kaposi’s sarcoma-associated herpesvirus (HHV-8/KSHV).

1.6 Clinical Pathology of HSV-1 and HSV-2

Viral infection in humans, the natural host, usually remains localized to cells of the epidermis and peripheral nervous system, except in newborn infants, who are more prone to disseminated infection. There are two serotypes of HSV, designated HSV-1 and HSV-2. HSV-1, which has an estimated prevalence of 70-90% worldwide (Roizman, 1993), is primarily associated with recurrent facial lesions in adults, but it also can cause encephalitis in certain individuals. HSV-2, with a prevalence of 20.8% worldwide (Aurelius, 1998) is primarily associated with severe recurrent genital lesions, but is also associated with neonatal infections. Infection with HSV-1 and to a lesser extent HSV-2 is the leading cause of infectious corneal blindness in North America, with over 550,000
cases annually. Worldwide, over 70% of people over the age of forty have antibodies against HSV-1 (Roizman and Sears, 1996).

HSV must come in contact with mucosal surfaces or abraded skin for infection to be initiated. Following viral replication at the site of infection, the viral nucleocapsid is transported via retrograde axonal flow by neurons to the dorsal root ganglia, where latency is established. If the virusreactivates from a latent to an active state, virus becomes evident at mucocutaneous sites, appearing as skin vesicles or ‘cold sores’. Primary infection can spread beyond the dorsal root ganglia, thereby becoming systemic; however this event is unusual. Such circumstances include disseminated neonatal HSV infection with multiorgan involvement, multiorgan disease of pregnancy, and rarely, dissemination in severely immunosuppressed patients. Therefore, these individuals can include patients receiving chemotherapy or radiation therapy (Saral 1990). In such immunocompromised patients, recurrent oral HSV infections can be severe with extensive local ulcerations that cause considerable pain and offer portals of entry for superinfection with bacteria and fungi (Epstein 1990). To date, there have been some cases of reactivation of orolabial HSV during radiotherapy of the head and neck.

Wild-type HSV is capable of replicating in both neurons and glia, resulting in necrotizing encephalitis and widespread hemorrhagic necrosis usually localized to the inferiomedial portion of the temporal lobe. However, it is not known why herpes simplex encephalitis, which is the most serious neurological complication of HSV-1, occurs so rarely (with a prevalence of ~1-2 cases per million per year) against a background of an almost ubiquitous asymptomatic infection in normal individuals (Kennedy 1984).

1.7 HSV Structure

The members of the Herpesviridae family are morphologically similar. The HSV particle consists of four components (Figure 1. 3); (i) an electron-opaque core, which contains the ~150 kb dsDNA genome, (ii) an icosahedral capsid surrounding the core, (iii) an amorphous tegument surrounding the capsid, consisting of virus-encoded regulatory proteins that are critical for the establishment of infection and transcription of early genes, and (iv) an outer envelope studded with virally-derived glycoproteins. Ten envelope glycoproteins appear as spikes on the virus surface when viewed by electron
microscopy: glycoprotein B (gB), gC, gD, gE, gH, gI, gJ, gK, gL, and gM, of which only gB, gD, gH, gK and gL are essential for productive infection in cultured cells (Table 1. 2). These glycoproteins are involved in the processes of virus entry, egress, cell-to-cell spread and immune evasion (Roizman and Sears, 1996).
Figure 1.3 Electron micrograph of the HSV particle (x 100,000).

The HSV virion consists of a double-stranded (ds) linear DNA genome contained within an icosahedral capsid (A). Surrounding the capsid is the amorphous tegument (B). The virion envelope (C) contains glycoprotein spikes, which are indicated by the arrows. This image was obtained from the University of Cape Town website (http://www.uct.ac.za/depts/mmi/stannard/emimages.html), and originally published by Stannard and colleagues in 1987 (Stannard, Fuller et al. 1987).
Table 1.2 Modifications and functions of the HSV-1 envelope glycoproteins.

<table>
<thead>
<tr>
<th>HSV-1 glycoprotein</th>
<th>Essential for replication in cell culture?</th>
<th>Post-translational modifications</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB</td>
<td>Yes</td>
<td>- N-linked oligosaccharides - homodimer</td>
<td>Binding; penetration; cell-to-cell spread; neuroinvasive determinant</td>
</tr>
<tr>
<td>gC</td>
<td>Yes</td>
<td>- N-linked oligosaccharides - O-linked oligosaccharides - homotetramer</td>
<td>Binding; C3b receptor</td>
</tr>
<tr>
<td>gD</td>
<td>Yes</td>
<td>- N-linked oligosaccharides - O-linked oligosaccharides - homodimer</td>
<td>Stable attachment; penetration; cell-to-cell spread; neuroinvasive determinant</td>
</tr>
<tr>
<td>gE</td>
<td>No</td>
<td>- heterodimer with gI</td>
<td>Cell-to-cell spread; Fc receptor</td>
</tr>
<tr>
<td>gG</td>
<td>No</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>gH</td>
<td>Yes</td>
<td>- heterodimer with gL</td>
<td>Penetration; egress; cell-to-cell spread</td>
</tr>
<tr>
<td>gI</td>
<td>No</td>
<td>- heterodimer with gE</td>
<td>Cell-to-cell spread; Fc receptor</td>
</tr>
<tr>
<td>gJ</td>
<td>No</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>gK</td>
<td>Yes</td>
<td>Egress</td>
<td></td>
</tr>
<tr>
<td>gL</td>
<td>Yes</td>
<td>- heterodimer with gH - proteolytic cleavage of transmembrane region</td>
<td>Penetration; cell-to-cell spread</td>
</tr>
<tr>
<td>gM</td>
<td>No</td>
<td>Possible cell-to-cell spread</td>
<td></td>
</tr>
</tbody>
</table>
1.8 *A Brief Overview of the HSV Life Cycle*

The viral replication cycle consists of two distinct phases; the lytic phase in which progeny virus is produced and the host cell is destroyed, and the latent phase in which the genome is able to remain in a quiescent episomal form for years until an unknown stimulus reactivates the virus and causes a lytic infection.

1.9 *The Lytic Cycle*

The HSV replication cycle is relatively short, lasting approximately 18 h, and results in the production of infectious viral progeny and the destruction of the host cell (Ward and Roizman 1994). In cell culture, infected cells become round in appearance and lose their adhesive properties. Other cytopathic effects include degradation of cell nucleoli and chromatin.

The virus life cycle begins when the virus recognizes a suitable receptor on the outside of a potential target cell and attaches to the receptor to initiate the process of infection. Fusion of the envelope with the plasma membrane rapidly follows initial attachment. The capsid is released into the cytoplasm and transported to the nuclear pores. The cellular cytoskeleton likely mediates the transport of HSV-1 capsids to nuclear pores, since drugs such as nocodazole, which disrupts microtubules, have been shown to inhibit neuritic transport of HSV-1 (Dales and Chardonnet 1977; Kristensson, Lycke et al. 1986). Once the virus reaches the nuclear pores, capsid uncoating occurs. The linear viral DNA is released into the nucleus (where transcription, replication of viral DNA, and assembly of new capsids take place) and immediately circularizes (Roizman and Sears 1996).

Two viral tegument proteins, virion host shutoff protein (VHS) and VP16, are released into the cell cytoplasm concomitant with viral entry. These proteins create an environment that is favorable for viral gene expression (Ward and Roizman 1994). VHS mediates the degradation of host cell mRNA, and in this manner downregulates host protein synthesis (Read and Frenkel 1983; Kwong and Frenkel 1987). VP16 acts *in trans* to initiate the transcription of viral genes (Batterson and Roizman 1983; Campbell, Palfreyman et al. 1984). HSV gene expression is coordinately regulated and occurs
sequentially in a cascade fashion (Roizman and Sears 1996). VP16 stimulates expression of a set of immediate-early proteins that then activate a series of early genes required for DNA synthesis. These early gene products in turn activate a set of late genes required for viral packaging. These early events in the life cycle cause a 'reprogramming' of the cell so that it becomes, in essence, a factory for making new HSV particles. During this time, the virus pirates various components of the host cells to ensure that it has all of the precursor molecules and machinery required to generate new virus particles. Moreover, several viral genes are expressed to protect the infected cell from immune destruction and apoptosis prior to the assembly of new virus particles. In this way, HSV has evolved to ensure that it can reproduce in a human host and spread from person to person in an efficient manner.

Viral DNA is transcribed throughout the reproductive cycle by host RNApolII, with concomitant participation of viral transcription factors. Viral gene expression is coordinately regulated via tegument proteins that promote the transcription of a sequentially ordered cascade of "immediate-early", "early", and "late" gene products. Meanwhile, viral DNA replication is carried out by a rolling circle mechanism, yielding concatemers that are cleaved into monomers and packaged into capsids (Roizman and Sears 1996).

Assembly occurs in two stages. After packaging of DNA into pre-assembled capsids, the virus matures and acquires infectivity by budding through the inner lamella of the nuclear membrane (Darlington and Moss 1968). After leaving the inner nuclear membrane, the route of egress of infectious virus particles is uncertain, but clearly involves the host cell secretory apparatus. This entire reproductive cycle occurs in approximately 18 to 20 h (Figure 1.4) (Roizman and Sears 1996).
Figure 1.4 Schematic representation of the HSV reproductive cycle.

(1) HSV attachment to cell surface receptors is followed by (2) fusion of the virion envelope with the cell membrane (penetration). (3) The nucleocapsid and two tegument proteins, VHS and VP16, are released into the cell cytoplasm. (4) The nucleocapsid is transported to nuclear pores where the viral genome is released into the nucleus and circularizes. VP16 initiates transcription of the $\alpha$ genes. Then $\beta$ genes are transcribed, whose protein products are necessary for (5) DNA replication. Concomitant with DNA replication is transcription of the $\gamma$ genes, which encode for the capsid proteins and glycoproteins. (6) DNA is packaged into preformed capsids. (7) Nucleocapsids acquire an envelope by budding from glycoprotein-modified patches on the inner nuclear membrane. (8) Enveloped viruses travel through the ER and Golgi apparatus, at which time the glycoproteins are modified. (9) Mature enveloped virions are transported to the cell membrane in vesicles and are then released (10) into the extracellular space. See text for details.
1.10 **HSV Latency**

Much of the clinical disease arising from HSV infection is due to the ability of the virus to interact with neurons of the peripheral nervous system. This allows the virus to survive throughout the life of the infected individual in a latent state and evade the host immune response. Following primary infection and replication at peripheral sites, virus attaches to and enters sensory nerve terminals (Vahlne, Nystrom et al. 1978). HSV particles travel along axons to neuronal cell bodies within the sensory ganglia (McLennan and Darby 1980). Latent virus is reactivated by hormonal imbalance, emotional stress or injury to tissues that are innervated by neurons that are latently infected with the virus. Despite extensive research however, the mechanisms by which the virus establishes and maintains a latent state or is reactivated, remain poorly understood (Whitley, Kimberlin et al. 1998; Efstathiou, Field et al. 1999).

1.11 **HSV Entry**

HSV infects a broad range of cells and thereby is able to cause disease in many tissues from diverse animal species. This relatively broad host range suggests that cellular receptors for HSV may be common to many different cell types and/or that there may be more than one pathway by which the virus can enter a cell. Since the viral envelope houses ten different viral glycoproteins, it seems likely that binding of the virus to a cell and the subsequent steps leading to virion-cell fusion may require sequential or simultaneous interactions between multiple virion proteins and several different receptors on the cell surface.

The process of HSV entry into the host cell can be divided into three separate events; (i) a reversible initial attachment to cell glycosaminoglycans, (ii) stable attachment to a protein receptor on the cell surface, (iii) penetration by fusion of viral and cellular membranes (Figure 1. 5).
**Figure 1.5** Schematic diagram of the process of HSV entry.
Adapted from McClain and Fuller, 1994 (McClain and Fuller 1994).
1.11.1 Binding

HSV uses HS, a glycosaminoglycan composed of long chains of alternating disaccharides of GlcNAc and IdoA/GlcA attached to a protein core, as an initial receptor prior to interaction with a secondary protein receptor for stable attachment. HS is widely expressed on the surface of human tissues, and is frequently altered in cancer cells (Damiens, El Yazidi et al. 1998; Jayson, Lyon et al. 1998; Kleeff, Ishiwata et al. 1998; Lambrecht, Le Bourhis et al. 1998; Parthasarathy, Gotow et al. 1998) (Jacobs, Julian et al. 1997) (Nackaerts, Verbeken et al. 1997). Thus the initial binding of HSV to the cell surface is mediated by interaction of viral envelope glycoproteins with HS and/or chondroitin sulfate (CS) GAG chains of cell surface proteoglycans (Spear 1993). Evidence for the interaction of HSV with GAGs stems from studies in which the cell surface HS is reduced by enzymatic digestion, resulting in a reduction in HSV infection (WuDunn and Spear 1989). In addition, animal cell mutants with defects in GAG synthesis all show some degree of resistance to HSV infection (Shieh, WuDunn et al. 1992; Gruenheid, Gatzke et al. 1993; Banfield, Leduc et al. 1995). Moreover, HSV infection is reduced by over 90% when soluble heparin or HS are present during inoculation (Gruenheid, Gatzke et al. 1993), and soluble dermatan sulfate (chondroitin sulfate B) has the ability to inhibit HSV-1 infection of both CS and HS/CS expressing mouse cell lines (Banfield, Leduc et al. 1995). The functional role of HS in HSV infection appears to be to concentrate viral particles at the cell surface, thereby increasing the frequency of a subsequent interaction with its secondary receptor (Johnson and Ligas 1988).

Although the presence of soluble GAGs reduces HSV infection, they do not permanently inhibit the infectivity of the virion. Therefore, it appears that the soluble GAGs act as competitive inhibitors to viral attachment. Experiments employing a biosensor system to observe the real-time binding of a protein to an immobilized ligand have shown that complexes between HSV-2 glycoprotein B and GAGs are reversible (Williams and Straus 1997). This leads to speculation that interaction between viral glycoproteins and cell surface GAGs may be sufficiently strong to promote binding to the cell surface, but still weak enough to allow release of the virion if viral entry does not
occur within a given time frame. Supporting evidence for this model of temporary virus adsorption to the cell surface is provided by the study of GAG deficient cell lines. In these cells, exogenous DS (a GAG analog) is able to form an alternative adsorption matrix for HSV-1 binding in the absence of other GAGs (Dyer, Banfield et al. 1997).

Several studies involving the alphaherpesviruses, HSV-1, BHV-1, and PrV were undertaken to identify the virion component responsible for initial binding to cell surface GAGs. The involvement of HSV-1 gC in virion attachment to host cells is supported by several lines of evidence. Deletion of the gC coding genes of HSV-1, BHV-1, or PrV results in mutants that are significantly impaired in their ability to bind to cells (Schreurs, Mettenleiter et al. 1988; Zuckermann, Zsak et al. 1989; Herold, WuDunn et al. 1991; Liang, Bbiuk et al. 1991). In addition, the gC homologs from each of these viruses bind to heparin affinity columns under physiological salt concentration (Schreurs, Mettenleiter et al. 1988; Mettenleiter, Zask et al. 1990; Sawitsky, Hampl et al. 1990; Okazaki, mitsuzaki et al. 1991) (Herold, WuDunn et al. 1991). Moreover, neutralizing antibodies specific for the gC homolog of each of the three viruses can block binding of virus to host cells (Fuller and Spear 1985; Zuckermann, Zsak et al. 1989; Okazaki, mitsuzaki et al. 1991; Svennerholm, Jeansson et al. 1991), while most neutralizing antibodies specific for other glycoproteins only block viral penetration. As well, heparitinase treatment of cells, which removes any cell surface HS GAGs, reduces the attachment of soluble gC-1 by 50% (Tal-Singer, Peng et al. 1995). Finally, removal of the heparin-binding domains of gC-1 significantly reduces the attachment efficiency of virus to cells (Trybala, Bergstrom et al. 1994; Tal-Singer, Peng et al. 1995). These studies strongly suggest that the primary interaction responsible for virus binding to the cell surface is between gC and HS.

However, an inconsistency with the role of gC in virus binding is the fact that it is not essential for infection (Langeland, Oyan et al. 1990; Herold, WuDunn et al. 1991; Sears, mcGwire et al. 1991; Laquerre, Argnani et al. 1998). Virions devoid of gC-1 are still infectious, although less efficient in attachment. Experiments showing that infection by gC-deficient HSV-1 can be inhibited by soluble heparin suggest that other viral glycoproteins may participate in the initial GAG binding event (Herold, WuDunn et al. 1991). This prompted Herold and colleagues (1994) to investigate whether there was a gC-independent mode of binding. The ability of HSV-1 gC-negative virus to bind to
cells devoid of HS was found to be significantly reduced compared to wild-type virus, indicating that gC independent binding of HSV-1 requires cell surface HS. Moreover, HSV-1 virions lacking both gC and gB are far more impaired in binding to cells than are virions that lack only gC. These data, along with the finding that gB has an affinity for heparin under physiological conditions, indicated that gB may also have a role in the initial binding of viruses to the cell surface (Herold, Visalli et al. 1994).

1.11.2 Stable attachment

It is now well established that binding of HSV to GAGs is followed by a stable form of attachment to a second non-GAG receptor. Early evidence for a secondary receptor came from the study of Chinese hamster ovary (CHO) cells and swine testes (ST) cells that were highly resistant to HSV infection, despite expressing normal amounts of HS (Subramanian, McClain et al. 1994). This indicated that these cells lacked a functional non-HS receptor required for efficient viral entry. This was further supported by the finding that sog9 cells, despite being GAG-deficient, are still susceptible to HSV infection (Banfield, Leduc et al. 1995).

While gB and gC are associated with initial binding to cell surface GAGs, it appears that HSV gD facilitates stable attachment. Functional gD is essential for entry, since gD-negative virions and virions treated with anti-gD neutralizing antibodies fail to enter cells (Fuller and Spear 1987). Glycoprotein D mediates infection in part by binding to a cell surface receptor. This is illustrated by the ability of UV-irradiated, gD-bearing virions or soluble gD to block infection by subsequently added virus in a saturable manner (Addison, Rixon et al. 1984; Johnson and Ligas 1988; Johnson and Spear 1989). Moreover, cells that constitutively express gD are resistant to infection by HSV in a process called gD-mediated interference (Campadelli-Fiume, Arsenakis et al. 1988; Johnson and Spear 1989; Campadelli-Fiume, Qi et al. 1990; Spear 1993). It was suggested that the excess amount of cell surface gD sequesters the endogenous stable attachment receptor and thereby prevents them from binding to gD on the virus, thus preventing infection (Campadelli-Fiume, Arsenakis et al. 1988; Johnson and Spear 1989; Campadelli-Fiume, Qi et al. 1990; Dean, Terhune et al. 1994). Johnson and colleagues demonstrated that binding of soluble gD can be reduced by treating cells with proteases
but is unaffected when cell surface HS is removed (Johnson, Burke et al. 1990). Thus the gD receptor is not an HS moiety. Structural data for HSV-1 gD demonstrate that it appears as a homotetramer containing a pronounced pocket in the center of the molecule (Pilling, Rosenberg et al. 1999).

The mannose-6-phosphate receptor (M6PR) was an early candidate for a gD-binding stable attachment receptor. M6PRs are found on the surfaces of cells primarily in clathrin-coated pits (Dahms, Lobel et al. 1989). Soluble gD-1 bound to M6PR of most mammalian cell surfaces. Moreover, the authors observed that the oligosaccharide fraction of glycoprotein D-1 (gD-1) is modified by mannose-6-phosphate (M6P) residues (Brunetti, Burke et al. 1994). This group subsequently demonstrated that soluble forms of the M6PRs could inhibit HSV-1 entry into monkey cells (Brunetti, Burke et al. 1995). However, susceptible mouse cells lacking M6PRs are still susceptible to infection (Brunetti, Burke et al. 1995). Furthermore, it was shown that antibodies directed against M6PR and a soluble form of the receptor block infection only to a small degree. These data suggest that M6PRs are not essential for entry in all cell types, and thereby their candidacy as a stable attachment receptor for HSV has been largely disregarded.

1.11.2.1 HveA/HVEM/TNFRSF14

The data summarized above provided strong evidence for at least one other receptor involved in HSV entry. To determine the identity of this receptor, Montgomery and colleagues (1996) transfected a human cDNA expression library into CHO cells, which express GAGs but are resistant to entry of HSV-1, PrV, and BHV-1 (Shieh, WuDunn et al. 1992; Warner, Geraghty et al. 1998). One clone, which encoded a member of the TNF receptor family, restored full susceptibility to HSV-1 infection in the CHO cells upon transfection (Montgomery, Warner et al. 1996). Sequencing of the cDNA revealed that it encoded a 283 amino acid protein, with three complete and one partial cysteine-rich repeat characteristic of members of the tumor necrosis factor/nerve growth factor receptor family. The protein was designated Herpesvirus entry mediator (HVEM), later renamed Herpesvirus entry mediator A (HveA), and officially named tumor necrosis factor receptor superfamily 14 (TNFRSF14) to indicate its place in an established family of proteins. HveA-transfected CHO-K1 cells also became susceptible
to HSV-2, but not to the related PrV, indicating that HveA is not a general mediator of alphaherpesvirus entry.

Upon this finding, a number of experiments were conducted to validate TNFRSF14 as a stable attachment receptor for HSV. Montgomery and colleagues (1996) found that the absence of the receptor significantly inhibited infection and that antibodies to the receptor blocked infection (Montgomery, Warner et al. 1996). Additional experiments have shown that truncated soluble forms of HveA could bind directly to HSV-1 gD (gD-1) in vitro, in a molar ratio of 2:1 (Whitbeck, Peng et al. 1997).

In addition, Montgomery and colleagues (1996) observed that anti-HveA antibody prevented HSV-1 infection of human T lymphocytes but not other human cell types (Montgomery, Warner et al. 1996). Although HSV primarily infects cells of epithelial and neural origin, several reports had described the replication of HSV in activated T cells (Pelton, Imrie et al. 1977; Rinaldo, Richter et al. 1978; Teute, Braun et al. 1983) and the presence of infected lymphocytes in biopsies of cutaneous lesions (Boddingius, Dijkman et al. 1987). HveA is expressed in many tissue types, including lung and liver, but it is expressed most abundantly in lymphoid organs (Marsters, Ayres et al. 1997). Taken together, these results raised the possibility that infection of T cells by HSV could serve as an anti-immune response mechanism. The finding that the normal cellular ligands for HveA are secreted lymphotoxin α and a new cytokine called LIGHT further supported this hypothesis (Mauri, Ebner et al. 1998). LIGHT refers to its homology to lymphotoxins, exhibition of inducible expression, and that it competes with HSV glycoprotein D for HveA, a receptor expressed on T lymphocytes. The fact that LIGHT is able to block HveA-dependent HSV-1 infection of T lymphocytes indicates that gD is a membrane-anchored virokine (a viral cytokine) that mimics LIGHT. A number of viruses, including adenovirus (Shisler, Yang et al. 1997), hepatitis C (Zhu, Khoshnan et al. 1998), as well as the herpesviruses EBV (Mosialos, Birkenbach et al. 1995) and HHV-8/KSHV (Bertin, Armstrong et al. 1997), target the TNFR for immunomodulation. Taken together, these interactions suggest an attractive model for HSV-1 infection of activated T lymphocyte whereby the virus, through gD, modulates HveA signaling activities, perhaps blocking extracellular signals for apoptosis. The TNF-related cytokine-receptor systems feed into signaling pathways involved in either cell death or
cell survival. Thus HSV gD-1 may be able to modify HveA-signaling activities during viral entry, which may in turn aid in virus survival and persistence.

1.11.2.2 HveB/Prr2/nectin-2α

In addition to human lymphoid cells, HSV can also infect cells of neuronal and epithelial origin. Therefore other receptors must exist for HSV entry. The finding that certain mutant strains of HSV-1 are unable to use HveA as a stable attachment receptor spurred yet another search for an HSV receptor. By repeating the screening of a human cDNA library for HSV-entry mediating cDNAs, using the mutant HSV-1 KOSrid1 as the selective agent, a single cDNA was isolated that could confer susceptibility to infection (Warner, Geraghty et al. 1998). This cDNA could mediate infection of several mutant strains of HSV-1, wild type HSV-2, and PrV into CHO cells. However, it failed to support entry of wild type HSV-1 or BHV-1. Sequencing of this cDNA revealed that it was identical to a previously described member of the immunoglobulin superfamily, poliovirus receptor related protein-2 (Prr2) (Eberle, Dubreuil et al. 1995). This protein was designated Herpesvirus entry mediator B (HveB), indicating its newfound function. Recently, Prr2 was renamed as nectin-2α (Takahashi, Nakanishi et al. 1999) and further found to function as cell-cell adhesion molecules (Aoki, Koike et al. 1997; Takahashi, Nakanishi et al. 1999).

HveB/Prr2 is expressed in human neuronal cell lines, fibroblastic cells, keratinocytes, and like HveA, primary activated T lymphocytes. However, antibodies to HveB did not block infection of many of these cells. The observed differences in the ability of HSV-1 and HSV-2 strains to use HveB may account, in part, for differences in viral tissue tropisms and pathogenicity.

1.11.2.3 HveC/Prr1/nectin-1γ and HveD/Pvr/CD155

Neither HveA nor HveB function as a co-receptor of both serotypes into epithelial cells, the mucosal epithelia being the site of initial replication. Moreover, neither HveA nor HveB can serve as a co-receptor for the other alphaherpesviruses, PrV and BHV-1. For these reasons, Geraghty and colleagues (1998) set out to isolate a common co-receptor for HSV-1, HSV-2, PrV, and BHV-1 (Geraghty, Krummernacher et al. 1998). Considering the ability of HveB/Prr2 to act as a stable attachment receptor for HSV, the
next logical step was to test the Prr2 homologs, Poliovirus receptor (PVR) (Mendelson et al., 1989) and poliovirus related receptor-1 (prr1) (Lopez et al., 1995). In their study, they identified poliovirus receptor-related protein 1 (prr1) protein (Lopez, Eberle et al. 1995), designated as herpesvirus entry protein C (HveC) as being able to mediate entry of all these viruses into resistant CHO cells. Prr1 was later renamed as nectin-1γ. Additionally, they demonstrated that PVR itself mediates BHV-1 and PrV infection, but not HSV infection. PVR was named Herpesvirus entry mediator D (HveD) to indicate its new role. An isoform of HveC was later identified and designated the herpes Ig-like receptor (HIgR). HIgR is able to mediate HSV-1, HSV-2, and BHV-1 infection; HIgR was renamed as nectin-1α. All of these receptors have been found to interact with HSV gD (Cocchi, Menotti et al. 1998).

Despite their abilities to act as herpesvirus entry mediators, HveA and HveB do not display the tissue distribution necessary to mediate infection of the epithelial and neural tissues that HSV normally infects. Significantly, HveC and HIgR are expressed in high levels in human epithelial and neuronal cells. As such, they are strongly favored as the prime candidate for the secondary receptors that allow both HSV-1 and HSV-2 to infect epithelial mucosa and spread to the nervous system (Cocchi, Menotti et al. 1998; Geraghty, Krummernacher et al. 1998). Clearly, different HSV-1 and HSV-2 strains vary in their ability to utilize HveA, HveB, HveC and HIgR for infection. This, and the fact that these receptors are expressed in distinct cell types will in part determine the pattern of viral spread in an infected individual.

1.11.2.4 Highly 3-O-sulfated heparan sulfate

The previously identified gD-binding receptors for HSV, HveA, HveB, and HveC are all cell surface proteins, encoded by cDNAs isolated from a human cDNA library. By screening a mouse cDNA library for the ability to mediate entry into CHO-K1 cells, Shukla and colleagues isolated a cDNA closely related to the human gene 3-O-sulfotransferase 3B (3-OST-3B) (Shworak, Liu et al. 1997; Shukla, Liu et al. 1999), which modifies HS late in biosynthesis (Lindahl, Kutsche-Gullberg et al. 1998). CHO-K1 cells normally display a full complement of HS and CS, which HSV uses for initial attachment via gC and gB (Shieh, WuDunn et al. 1992). However, HSV entry is blocked
due to a lack of stable attachment receptors. Expression of 3-OST-3B in these cells results in the generation of highly sulfated disaccharide repeats in the HS polymer (2-O-sulfated iduronic acid and 3-O-sulfated/6-O-sulfated glucosamine). By engaging these molecules, HSV was able to bind more efficiently to the primary receptor (Liu, Shriver et al. 1999; Shukla, Liu et al. 1999).

Several lines of evidence demonstrated that HSV-1 entry mediated by 3-OST-3B is dependent upon gD and on the generation of gD-binding sites in HS. First, HSV mutants with single amino acid substitutions in gD were unable to infect cells expressing 3-OST-3B (Shukla, Liu et al. 1999). Second, both membrane-bound and soluble gD competed with virus for the receptors generated by 3-OST-3B-modified HS. Finally, pretreatment of surfaces of 3-OST-3B-expressing CHO-K1 cells with HS-degrading enzymes (heparitinases) eliminated binding of soluble gD to the cell surface.

These results raise the possibility that HSV-1 entry into cells could be mediated entirely by HS, provided the appropriate sites for virus binding and gD binding were present in the HS. Unlike the HveA, B, and C stable attachment receptors, 3-OST-3B is widely expressed in human tissues, suggesting that in tissues that do not express any of the Hve-receptors, 3-OST-3B modifications of HS may compensate and permit virus entry.

1.11.3 Penetration

Enveloped viruses, such as HSV, can either enter cells by fusion with the plasma membrane or by endocytosis. Agents that block endocytosis do not inhibit HSV entry, which suggests that HSV fuses with the plasma membrane (Fuller, Santos et al. 1989). Nonetheless, electron microscopic evidence suggests that HSV may be able to enter by endocytosis as well (Morgan, Rose et al. 1968). However, HSV entry by endocytosis results in degradation off the virus and non-productive infection (Campadelli-Fiume, Arsenakis et al. 1988).

Studies using mutant viruses and monoclonal antibodies show that HSV-1 gB, gD, gH, gK, and gL are essential for infection (Cai et al., 1988; Ligas and Johnson, 1988; Fuller et al., 1989; Forrester et al., 1992; Roop et al., 1993; Hutchinson et al., 1995). HSV mutants deleted for any of these essential glycoproteins are able to attach to
permissive cells, but are unable to penetrate (Cai, Person et al. 1987; Ligas and Johnson 1988; Forrester, Farrell et al. 1992; Roop, Hutchinson et al. 1993). However, adsorbed virions can penetrate the cells to initiate infection in the presence of polyethylene glycol (PEG), a chemical fusogen. Furthermore, anti-gB, gD and gH neutralizing antibodies permit attachment to cells but prevent penetration (Fuller and Spear 1987; Fuller, Santos et al. 1989; Navarro, Paz et al. 1992). Co-expression of gL and gH is required for normal post-translational modifications, folding and intracellular transport of both glycoproteins (Gompels and Minson 1989; Roop, Hutchinson et al. 1993). Therefore an HSV-1 mutant deleted in gL is unable to incorporate gH into the virion envelope and enter into cells, although it could adsorb efficiently to the cell surface. These data suggested the involvement of these glycoproteins in fusion activity (Fuller and Spear 1987; Fuller, Santos et al. 1989).

Thus far, gB is the only confirmed fusogenic glycoprotein. Anti-gB monoclonal antibodies that inhibit viral penetration map to amino acid residues 241-441 in the gB molecule, a region that is centrally located within the ectodomain (Highlander, Cai et al. 1988). However, recent work with BHV-1 gB truncation mutants suggests that this domain may be present in the second of the three membrane spanning regions (Li, van Drunen Littel-van den Hurk et al. 1997). The precise location of the gB domains critical for viral penetration remains unclear.

Despite continual progress in this field, the molecular mechanisms underlying HSV penetration are not well understood. It is evident, however, that gB, gD, gH, gL, and gC can associate with one another in the virion envelope (Handler, Eisenber et al. 1996). Chemical cross-linking studies have shown that these glycoproteins can form homodimers and hetero-oligomers with each other (Handler, Cohen et al. 1996). This study also showed that complexes present during attachment disappeared as penetration proceeded. It is possible that during penetration, the glycoproteins undergo a conformational change or that the virion envelope undergoes a physical change (such as the formation of a fusion pore). Together, these findings support the concept that the HSV viral glycoproteins form a complex during entry. This is not surprising since all known virus fusion proteins are oligomers and many cellular fusion events are dependent on the formation of multi-subunit complexes (White 1990).
Recently, a study by Minson and colleagues demonstrated that the incorporation and arrangement of these glycoproteins in the virion envelope are independent of each other, and therefore the formation of a functional complex is unlikely (Rodger, Boname et al. 2001). Much of our current understanding of the functions of gB, gC, gD, gH, and gL comes from studies of deletion mutants lacking these proteins (Roizman and Sears 1996). The conclusions drawn from these studies are predicated on the assumption that the absence of one protein does not affect virion composition with respect to the others. Furthermore, the concept that these proteins are organized into a functional complex such that the absence of one member of the complex has no effect on the spatial arrangement of the others seems untenable. It could be that these molecules are arranged independently in the virion but are sufficiently closely packed that cross-linking between them can occur.

1.12 **HSV Egress**

The egress of herpesvirus particles from an infected host cell is believed to initiate when mature capsids attach to patches in the inner nuclear membrane that contain immature viral envelope glycoproteins. Capsids bud through the inner lamella of the nuclear membrane into the perinuclear space, acquiring an envelope (Darlington and Moss 1968). However, subsequent routes taken by these particles as they travel out of the cell remain unclear. One view is that enveloped virions move via the secretory pathway by vesicular transport from the endoplasmic reticulum (ER) to the Golgi complex and trans-Golgi network, ultimately reaching the plasma membrane where they are released. This mechanism of egress implies that the virion acquires a full complement of transmembrane glycoproteins at the inner nuclear membrane and that these immature glycoproteins are processed *in situ* during egress through the Golgi compartment. Evidence in favor of this model was reported by Johnson and Spear (Johnson and Spear 1982), who demonstrated that monensin, an ionophore that disrupts the budding of vesicles from the Golgi, inhibits the transport of HSV progeny virions to the cell surface. Treatment of HSV infected cells with monensin led to an accumulation of enveloped particles in what appeared to be Golgi-derived vacuoles. The view that
herpesvirus egress involves a single envelopment process has the intuitive virtue of economy, and this model finds favor in most standard texts.

An alternative model for egress involves the fusion of enveloped virus particles in the perinuclear cisternae with the outer nuclear membrane, thereby releasing naked nucleocapsids into the cell cytoplasm (Jones and Grose 1988; Whealy, Card et al. 1991; Gershon, Sherman et al. 1994; Zhu, Gershon et al. 1995). Capsids are often observed in herpesvirus-infected cells adjacent to membrane-bound vesicular structures, and it has been suggested that these represent virions in the process of re-envelopment by Golgi-derived vacuoles. Evidence for this model comes from a study by Whealy and colleagues (1991), who demonstrated that treatment of PrV-infected cells with brefeldin A, an inhibitor that destroys the Golgi apparatus, resulted in the accumulation of envelope-free capsids in the cytoplasm. Brefeldin A treatment of HSV-1 infected cells results in the accumulation of naked nucleocapsids in the cell cytoplasm (Cheung, Banfield et al. 1991). Moreover, Browne and colleagues demonstrated that restriction of the expression of glycoprotein H to the ER-inner nuclear membrane, by means of an ER-retention motif, resulted in the release of virus containing no detectable amounts of the glycoprotein (Browne, Bell et al. 1996). Recently, ER or Golgi gD targeting experiments have further supported this model of egress (Whiteley, Bruun et al. 1999; Skepper, Whiteley et al. 2001). Skepper and colleagues used immunogold electron microscopy to examine the distribution of gD in HSV-infected cells. They found that in cells infected with HSV-1 encoding an ER-retrieved gD, extracellular virions exhibited very little gold decoration when compared to perinuclear enveloped virions. These data suggest that the HSV-1 virion acquires an envelope from a subcellular component other than the ER-inner nuclear membrane.

However, arguments against this pathway include analysis of a mutation in HSV-1 glycoprotein D, which results in the accumulation of large numbers of unenveloped capsids in the cytoplasm. This mutation also causes reduced yields of extracellular virus. Virus egress depends on Golgi-dependent maturation of viral glycoproteins. Defects in glycoprotein processing block the transport of virus to the extracellular space. The process of virion translocation across the cytoplasm to the extracellular space is also regulated by various viral components. In a number of cell lines, the Golgi apparatus
fragments as a result of infection and therefore virions devoid of the HSV UL20 gene product get trapped in the perinuclear space (Baines, Ward et al. 1991; Campadelli-Fiume, Brandimarti et al. 1993). In addition to UL20, gH (Desai, Schaffer et al. 1988; Browne, Bell et al. 1996), gD (Campadelli-Fiume, Brandimarti et al. 1993), and gK (Hutchinson and Johnson 1995) are involved in the transport of enveloped virus. Virions accumulate within the cytoplasm when any one of these proteins is absent. Therefore it has been proposed that naked cytoplasmic nucleocapsids represent a dead-end rather than a stage in the route of virus egress (Campadelli-Fiume, Farabegoli et al. 1991).

1.13 Cell-to-Cell Spread of HSV

HSV spreads to new host cells by production of extracellular virions and direct cell-to-cell transmission. The production of extracellular HSV likely plays an important role in dissemination to other hosts. However, HSV antibodies do not restrict HSV spread in epithelial tissues, suggesting that direct cell-to-cell spread is an important property of HSV pathogenesis.

Spread of HSV by both the extracellular and cell-to-cell routes requires viral glycoproteins gB, gD, and gH-gL. If viruses deficient in these glycoproteins are grown on complementing cell lines (to provide the missing glycoprotein) the mutant viruses can enter cells but cannot spread to adjacent cells and form plaques (indication of viral spread) (Cai, Gu et al. 1988; Ligas and Johnson 1988; Forrester, Farrell et al. 1992; Roop, Hutchinson et al. 1993). Recently, cell fusion was found to depend also on the expression of cell surface entry receptors specific for gD (Pertel, Fridberg et al. 2001). The finding that the glycoproteins essential for spread are also required for viral entry suggests that they play similar roles in cell-to-cell spread as they do for entry. Indeed, there is evidence that the lysine-rich heparin-binding domain of gB, but not gC, facilitates direct cell-to-cell spread of HSV-1 in cultured cells (Laquerre, Argnani et al. 1998), while antibodies directed against gD, gH, and gL, prevented HSV-1 induced cell-cell fusion (Gompels and Minson 1986; Highlander, Sutherland et al. 1987).

Glycoproteins gE and gi form a hetero-oligomeric complex (Johnson and Feenstra 1987; Johnson, Frame et al. 1988), that aids HSV in the process of immune evasion by binding to the Fc region of human immunoglobulin G (IgG) causing IgG aggregation.
These glycoproteins are not required for entry or for the production of infectious progeny. However, there have also been reports that deletion of gE or gI from HSV-1 severely inhibited the cell-to-cell spread of virus both in cell culture and in mice (Dingwell, Brunetti et al. 1994; Dingwell, Doering et al. 1995). A recent study by Dingwell and Johnson (1998) demonstrated that the gE-gI complex co-localizes with cell junction components, supporting a model whereby gE-gI mediate transfer of virus to adjacent cells across cell junctions. This mode of transmission is particularly important to HSV pathogenesis, since the virus is therefore able to avoid neutralization by anti-HSV antibodies.

1.14 Glycosaminoglycan-Deficient Cells

A selection procedure to isolate HSV-resistant mouse cells (gro mutants) was developed to identify host cell factors that facilitate HSV infection (Tufaro, Snider et al. 1987). In this procedure, mouse L cells were infected with HSV-1 at an MOI of 1-3 PFU/cell, and after three days, any surviving cells were clonally expanded. This led to the isolation of the mutant cell line gro2C, which synthesizes CS but not HS. Gro2C cells were found to be 90% resistant to HSV-1 infection relative to parental control L cells (Gruenheid, Gatzke et al. 1993). The observation that gro2C cells were susceptible to HSV-1 infection, despite being deficient in HS GAGs, suggested that HSV-1 could interact with cell surface CS to mediate infection (Banfield, Leduc et al. 1995). This was supported by the observation that soluble CS inhibited HSV-1 infection on gro2C cells.

To uncover a cell line that was defective in additional components of the virus entry pathway, Banfield and colleagues (1995) repeated this selection procedure to select for a variant of gro2C cells even less susceptible to infection. The sog9 cell line was hence derived from gro2C cells and was nearly uninf ectible by HSV-1 (Banfield, Leduc et al. 1995). This observation can be explained by the finding that sog9 cells harbor additional defects in the GAG synthesis pathway such that no HS or CS GAGs are expressed on the cell surface. Importantly, HSV-1 infection of sog9 cells is not significantly reduced by soluble HS, indicating that infection of sog9 cells is GAG-independent. Thus GAGs do
not appear to be essential for HSV infectivity. However, in the absence of GAGs, the ability of the virus to bind the cell surface is significantly reduced.

1.15  

**Dextran Sulfate: A Glycosaminoglycan Analog**

During the study of GAG-deficient cell lines, it was found that HSV-1 infection could be restored by incubating these cells in a low concentration of DS (Banfield, Leduc et al. 1995; Dyer, Banfield et al. 1997). Dextrans are high molecular weight polymers of D-glucopyranose produced from sucrose by the bacterium *Leuconostoc mesenteroides* (Lindberg and Svenson 1968). Dextran sucrase catalyzes the transfer of glucosyl groups (D-glucose) from sucrose to the growing dextran chain, where they are linked by α1→6 linkages. Branching of the chain arises from α1→3 and from α1→4 linkages. Dextran fractions of different average molecular weight are obtained by partial hydrolysis. DS is a synthetic sulfated carbohydrate derived from the esterification of dextran (Figure 1.6). Partial hydrolysis and sulfation produces DS fractions of different average molecular weight (Witvrouw and De Clercq 1997). Thus, DS is a highly sulfated GAG analog and as such is a useful reagent for studying GAG-ligand interactions. For example, DS has been shown to be as active as heparin in binding fibronectin (Ruoslahti 1988). This activity of DS demonstrates that GAG-ligand interactions are not always specific and can be functions of charge density. Indeed, large glycosaminoglycan chains tend to interact more strongly with ligands than small ones (Klebe and Mock 1982).
Figure 1.6 Structure of the glycosaminoglycan analog dextran sulfate.
1.15.1 DS and viral entry

DS is an effective inhibitor of enveloped virus infection (Baba, Snoeck et al. 1988). DS can inhibit viral infection by a number of mechanisms. For example, DS causes certain strains of Coxsackie virus to aggregate, an effect which seems to inhibit plaque formation by repressing viral release (Totsuka, Mukoyama et al. 1981). Ohki and colleagues (1992) demonstrated that DS inhibits the binding and subsequent fusion of Sendai virus with erythrocyte ghosts. This may be due to the steric hindrance of a large polyanionic macromolecule adhered to the virion surface (Ohki, Arnold et al. 1992). DS has also been shown to inhibit HIV-1 binding, replication and formation of syncytia (Baba, Pauwels et al. 1988; Baba, Snoeck et al. 1988; Lederman, Gulick et al. 1989; Ida, Kurata et al. 1994). Along these lines, Callahan and colleagues (1991) observed that DS interacted with one of the HIV glycoproteins, gp120. This interaction inhibits binding of HIV to the cell surface, and forms the basis for new therapeutic strategies (Callahan, Phelan et al. 1991). Finally, DS inhibits HSV attachment and infection of cells (Banfield, Leduc et al. 1995). The inhibitory action of DS on the virus can be attributed, in part, to a reduction in electrostatic binding. The positively charged sites on the proteins are likely sequestered by negative sulfate groups on DS and are therefore unavailable to interact with cell surface HS. In light of these data, it was surprising that DS was found to enhance HSV-1 infection of GAG-deficient sog9 cells (Dyer, Banfield et al. 1997). This finding supported a model whereby in the absence of HS, HSV-1 can use DS as a surrogate receptor to mediate viral attachment and subsequent infection. Another study further upheld this model by showing that DS can mediate infection of otherwise resistant GAG-deficient mature muscle cells by HSV-1 (Yeung, Bockhold et al. 1999) (Chapter 3).

1.15.2 Clinical uses

Dextran has been used for decades as a clinical plasma volume expander, therefore its biocompatibility is well established. In fact, Dextran-hemoglobin was the first conjugate introduced as a blood substitute. Its selection as carrier polymer in a hemoglobin-based blood substitute is further suggested by its beneficial actions on blood flow. Furthermore, these molecules are not immunogenic in humans (Kabat and Bezer
1958). Dextran is completely metabolized or excreted from the body after brief storage in the cells of the reticuloendothelial system, and it can be chemically modified by a variety of methods to form defined and stable compounds.

Another clinically relevant use of dextran involves the treatment of anemia of chronic renal failure by the use of intravenous iron. Iron is delivered in the form of iron dextran to reduce toxicity and increase bioavailability. Until recently, the only commercial intravenous iron preparation approved by the Food and Drug Administration (FDA) for use in the United States was iron dextran, available as INFeD® (Schein Pharmaceutical, Inc., Florham Park NJ) and DexFerrum® (Luitpold Pharmaceuticals, Inc. and distributed by American Regent Laboratories, Inc., Shirley, NY). Since 1999, iron gluconate has also received FDA approval (Johnson, Mason et al. 1999).

Finally, dextran can be used in the prophylaxis of postoperative thromboembolic disorders. It has been shown to be as effective as heparin in the prophylaxis of fatal postoperative pulmonary embolism (Gruber, Saldeen et al. 1980).

1.15.2.1 Volume expander

The metabolic activity of living cells in all organs and tissues depends on adequate flow of blood through the capillaries and postcapillary venules where the essential exchange between blood and tissues is achieved. This is the "business end" of the circulation and virtually the reason for the existence of all of the circulatory system with its complex structure, and all its intricate neurohumoral regulatory mechanisms. Sluggish flow and stagnation of blood in the capillaries and venules are the central features and early signs of all forms of circulatory shock. It is also well known that replacement of lost volume with plasma substitutes is of no avail in alleviating shock and in reversing the progressive metabolic deterioration unless it serves to restore and maintain flow through the network of minute vessels everywhere. Hence the effects of expander agents and other forms of substitution therapy on the fluidity or viscosity of blood, when it encounters the near zero flow conditions in the minute vessels, can obviously be of critical importance.
1.15.2.2 Prolongation of the effects of hypertonic solutions

Hypertonic solutions have been shown to be superior to normotonic solutions in terms of restoration of hemodynamic state, improving tissue perfusion and increasing survival of patients with traumatic shock. Small volumes of hypertonic solutions, up to 4 ml/kg body weight, were more efficient than four to ten times more isotonic solutions (Maningas, Mattox et al. 1989). A solution of 6% dextran – 7.5% saline has been shown to be highly effective in normalizing cardiovascular function in patients with hemorrhagic shock, due to rapid mobilization of fluid from the extravascular compartment (Kramer, Perron et al. 1986). This solution might also have other beneficial local factors, such as reversal of organ edema, improvement of coronary blood supply, and a scavenging effect on toxic oxygen metabolites (Younes, Aun et al. 1992; McDaniel, Nguyen et al. 1994).

1.15.2.3 Drug carrier

The combination of water solubility, availability in a wide range of molecular sizes, lack of significant toxicity or tissue tropism, and stability renders dextran an excellent drug carrier among biodegradable polymers. In this light, dextran conjugation has been employed to stabilize the protein molecule and to prolong the plasma half-lives of asparaginase (Wileman, Foster et al. 1986), carboxypeptidase (Melton, Wiblin et al. 1987), adenosine deaminase (Rosemeyer, Kornig et al. 1982), and arginase (Sherwood, Baird et al. 1977). Special benefits include thermal stability, resistance to proteolytic digestion, prolonged half-life, and decreased immunogenicity (Burnham 1994). Furthermore, covalent conjugation of dextran to proteins increases the effective size of the molecule, prevents its renal excretion and slows down its disappearance from the circulation (Tam and Wong 1988). The cumulative effect of increasing persistence of an agent in circulation by dextran encapsulation is three-fold: (i) they can slowly accumulate (passive accumulation via an impaired filtration mechanism) in pathological sites with affected vasculature (tumors, inflammations); (ii) they can help achieve a better targeting effect for agents that need to reach pathological area with diminished blood flow; (iii) they protect the agent from undesirable interactions with biological milieu.
components. The coupling of proteins to dextran is therefore an important means to enhance drug delivery and consequently therapeutic efficacy.

1.15.2.4 Organ transplantation

The classic triad of problems associated with transplantation - technical, immune rejection, and procurement - is well known. The technical problems generally have been solved. The problem of immune rejection remains a serious barrier, but work in this area is progressing rapidly and new approaches are under trial. Greater attention is being given to tissue typing, the matching of the tissue types of the donor and the recipient, in an attempt to preclude severe reactions between graft and the host. With respect to the third problem, the logistics (procurement, preservation, and storage of organs) are constantly being improved. Currently, the method consists of hypothermia and hyperbaric oxygen, combined with continual organ perfusion. Dextran plays an important role in the perfusion process; it removes stagnant blood, prevents thrombosis, red blood cell aggregation and capillary obstruction, cools the organ so that rapid core temperature to suitable hypothermic levels is rapidly achieved, and introduces anticoagulants and cryophylactic agents if desired, as well as vasodilators and metabolic inhibitors (Derrick and Guest 1971).

1.15.2.5 Microcirculatory flow

Dextrans are routinely used to improve peripheral blood flow. While they act principally by plasma volume expansion, they also produce a decrease in blood viscosity. This promotes a subsequent improvement in flow and has a specific red blood cell disaggregating activity (Audibert, Donner et al. 1994). This has been effective in trauma patients, in whom microcirculatory blood flow deteriorates.

Dextran has also been shown to form a thick layer on the luminal surface of capillary endothelium, reducing the electrostatic charges. It may be that plasma flow could be facilitated by a reduced interaction of endothelial receptors with specific plasma proteins (Baldwin, Wu et al. 1991).
1.16 Vectors for Gene Therapy

Central to the concept of gene therapy is the ability to deliver exogenous nucleic acids to cells of various tissues. Although some success has been reported in the uptake of “naked” DNA, efficient delivery and persistent expression have been limited to only a few tissues such as muscle. To improve the efficiency and stability of gene delivery, numerous laboratories are evaluating both viral and nonviral vectors for gene transfer. Viruses have evolved to become highly efficient at nucleic acid delivery to specific cell types while avoiding immunosurveillance. Nonviral vectors such as liposomes are clearly nonpathogenic, but are also less efficient in the transfer of nucleic acid to the nucleus of cells.

1.16.1 The Advantages of Using HSV

HSV-1 has many attractive features for development as an oncolytic and gene transfer vector. First, it is large and well studied. HSV-1 is an enveloped virus containing approximately 152 kb of DNA. The essential and nonessential genes have been identified, and HSV genetic-engineering techniques have been developed (Roizman 1996). Theoretically, up to 30 kb of the genome can be replaced with foreign DNA yet still leave a virus capable of replicating in an appropriate cellular environment. Second, multiple genes associated with neurovirulence can be deleted without affecting the virus’ capacity to replicate within and destroy tumor cells during the normal lytic phase (Goldstein and Weller 1988; Chou, Kern et al. 1990; Roizman 1996). Third, antitherpetic agents are available and provide a safety mechanism in case undesired local or systemic infection occurs. Indeed, HSV-1 vectors can be made with increased sensitivity to such agents. Finally, HSV-1 does not integrate into the cellular genome (Roizman 1996), so it cannot cause insertional mutagenesis. Even during viral latency, HSV-1 persists in neurons as an episome.

Furthermore, HSV vectors have the advantages of being able to infect nondividing cells and establishing latency in some cell types. The ability to establish latency in neuronal cells makes HSV an attractive vector for treating neurological disorders such as Parkinson’s and Alzheimer’s. In addition, the ability of HSV to efficiently infect a range of different cell types, such as muscle and liver, may make it an
excellent vector for treating non-neurological diseases. Importantly, HSV is sensitive to
drugs such as acyclovir (ACV) and thereby can be adequately controlled.

HSV replicates by an ensuing cascade of gene expression. As mentioned earlier,
VP16, which is carried into the cells as part of the viral tegument, stimulates expression
of a set of immediate-early proteins that activate a series of early genes required for DNA
synthesis. These early gene products in turn activate a set of late genes required for viral
packaging. Thus to render HSV replication-defective, inactivation of one or more of
these immediate-early proteins (ICP0, ICP4, ICP22, and ICP27) results in a vector unable
to replicate, except in a complementing cell line (Marconi, Krisky et al. 1996; Wu,
Watkins et al. 1996; Zhu, DeLuca et al. 1996). Many HSV proteins are nonessential for
viral replication and thus, can be deleted to permit the insertion of large exogenous
coding regions into the viral genome.

One problem associated with HSV-based vectors has been the toxicity of the
vector in many cell types. The generation of HSV vectors with deletions in many of the
immediate-early gene products has resulted in vectors with reduced toxicity and
antigenicity, as well as prolonged expression in vivo. An alternative approach to
producing infectious HSV vectors is the use of amplicons (Neve and Geller 1995; Geller
1997; Horsburgh, Hubinette et al. 1999). In this approach, a plasmid containing an HSV
origin of replication and packaging sequence is co-transfected with either cosmids or a
bacterial artificial chromosome (BAC) containing the HSV genome that contain defective
packaging sequence. The resulting virus contains only plasmid sequences and thereby
toxicity associated with low-level HSV gene expression is eliminated. Although helper-
free stocks of virus can be generated, viral titers are still very low.

1.16.2 Relevance of Immune Responses to HSV

HSV immunity involves all arms of the host immune system: B cells, CD4+ and
CD8+ T cells, and NK cells (Brach, Bonneau et al. 1997; Morrison and Knipe 1997). By
adulthood, 60-90% of the population is seropositive for HSV (Whitley 1996). The host
immune response to a primary HSV infection in mice (Halford, Veress et al. 1997;
Walker and Leib 1998) and to a much lesser extent in humans (Ashley, Benedetti et al.
1985; Whitley, Kimberlin et al. 1998) confers a protective effect on subsequent HSV-1
infection. Infection of mice with HSV induces long-lived immunity, involving both T and B cells (Morrison and Knipe 1994; Morrison and Knipe 1997). *In vitro*, antibodies and leukocytes are effective in blocking or eliminating HSV infection (Lopez, Arvin et al. 1993).

Potential clinical recipients of HSV therapy will be immune-competent or immune-suppressed due to steroid treatment, and likely HSV seropositive. To address whether prior HSV exposure and seropositivity will be a confounding factor for this therapeutic approach, Chahlavi and colleagues examined these issues in animal models of cancer. Humoral and/or cell-mediated immune responses could neutralize the spread and cytopathic effect of HSV, thereby reducing therapeutic efficacy; alternatively, immunization could induce an inflammatory response in the tumor against HSV that could stimulate an antitumor immune response and thereby increase therapeutic efficacy. The findings indicated that in immune-competent animals, inoculation of tumors with HSV induced a systemic, tumor-specific immune response involving CD8\(^+\) cytotoxic T cells (Chahlavi, Rabkin et al. 1999).

A number of studies using live HSV vaccination in different mouse models suggests that anti-HSV antibody principally limits viral invasion of the CNS, whereas T cells, particularly CD4\(^+\), inhibit primary viral replication and protect against latent infection (Mitchell and Stevens 1996; Morrison and Knipe 1997). However, the host immune response does not seem to be sufficient to block acute viral replication. In humans, recurrences of HSV infections occur frequently, even in the presence of high antibody levels and cellular immunity (Corey, Reeves et al. 1978; Zweerink and Stanton 1981; Posavad, Koelle et al. 1996). In vaccinated mice, under conditions of protective immunity, viral replication at the site of HSV infection is high in the first couple of days after challenge (Morrison and Knipe 1994). Such acute viral replication may be sufficient to elicit the antitumor response in seropositive mice. Therefore the studies undertaken by Chahlavi and colleagues suggest that the HSV immune status of patients is unlikely to affect HSV tumor therapy (Chahlavi, Rabkin et al. 1999).
1.17 **Using HSV as Delivery Vectors**

Advances in molecular virology, and in understanding the molecular basis of human disease, has led to intensive efforts to use viruses for delivering therapeutic genes to cells. Although gene therapy to treat disease is still in its infancy, the techniques developed for gene delivery to cells have become more sophisticated, and are useful in a variety of experimental settings. Vectors have been developed that are reasonably nontoxic and easy to manipulate in the laboratory.

1.17.1 **Skeletal Muscle**

As the molecular basis of an expanding number of inherited disorders has been discovered, increasing focus has been placed on gene therapy as a potential approach for the treatment of patients. The transfer of a functional gene into a particular tissue to alleviate a biochemical deficiency has been explored in many disease systems using a variety of gene delivery approaches. Human inherited disorders of muscle comprise some of the most common diseases of childhood. Hence, skeletal muscle has been primarily studied as a target tissue for the delivery of genes encoding proteins that may be therapeutic for inherited muscle disorders. However, since the multi-nucleated and post-mitotic myofibers in skeletal muscle are capable of both long-term transgene expression and secretion of proteins into circulation, gene delivery to skeletal muscle has also been investigated as a means to create a tissue reservoir for the secretion of non-muscle proteins such as growth hormone (Barr and Leiden 1991; Dhawan, Pan et al. 1991), factor IX (Dai, Roman et al. 1992; Yao and Kurachi 1992; Baru, Sha'anani et al. 1995; Wang, Zheng et al. 1996), erythropoietin (Hamamamori, Samal et al. 1994; Hamamori, Samal et al. 1995), and kallikrein (Xiong, Chao et al. 1995).

1.17.1.1 **Limitations for muscle-directed gene therapy**

Recent research has increasingly been focused on the application of gene delivery using viral vectors. However, these strategies have been limited by several factors including cytotoxicity, immune-rejection, poor efficiency, and inability to infect mature myofibers (Acsadi, Dickson et al. 1991; Johnson, Miyanohara et al. 1992; Acsadi, Jani et al. 1994; Yang, Nunes et al. 1994; Huard, Goins et al. 1995; Huard, Krisky et al. 1997).
Novel mutant vectors have reduced the problems associated with viral cytotoxicity and immune rejection (Kochanek, Clemens et al. 1996). However, the inability of viral vectors to efficiently transduce mature muscle fibers has remained a major barrier to the application of gene delivery to skeletal muscle.

1.17.1.2 The post-mitotic state of muscle cells

Skeletal muscle cells differentiate into myofibers and become post-mitotic early in development, which is a major barrier to the application of retroviral vectors in particular. Retrovirus is known to require dividing cells, such as myoblasts, for genomic integration and gene expression (Salvatori, Ferrari et al. 1993). However, HSV vectors are capable of transducing post-mitotic, immature myofibers in vitro and in vivo (Huard, Feero et al. 1996; Huard, Krisky et al. 1997). Therefore, other factors are involved in the poor level of HSV transduction of mature myofibers.

1.17.1.3 The development of the extracellular matrix into a physical barrier

A major barrier has been found to be the extracellular matrix, which appeared to act as a physical barrier to viral transduction of mature myofibers. The extracellular matrix, which is the connective tissue that surrounds mature skeletal muscle fibers to support mechanical properties, muscle regeneration, and synaptic interactions at the neuromuscular junctions, was found to contain pores with an estimated diameter of 40 nm (Yurchenco 1990). Thus, theoretically, these pores are too small to allow penetration of the extracellular matrix by HSV particles (approximately 120 nm) (Roizman and Furlong 1974). By using immunohistochemistry to co-localize collagen IV and HSV particles, Huard and colleagues (1996) showed that the viral particles were blocked by the extracellular matrix and remained in between the myofibers (Huard, Feero et al. 1996). In addition, higher levels of HSV transduction were observed in muscle from mature dy/dy mice that exhibit myofibers carrying an underdeveloped extracellular matrix, as compared to the levels detected in muscle from normal, age-matched mice (Huard, Feero et al. 1996). The transducibility of immature muscle by HSV can be explained by the fact that the extracellular matrix surrounding the immature myofibers has a poorly developed structure that allows viral penetration.
1.18 Using HSV as Oncolytic Vectors

Most clinical protocols involving gene therapy are directed against cancer because of the disease's lethality and the poor prognosis for many cancer patients prescribed conventional drug or radiation treatment. Many different approaches for treating cancer have been tried both in animal tumor models and now in the clinic.

1.18.1 Cancer Gene Therapy: Replication-Defective and Replication-Competent Vectors

Viral vectors used for cancer therapy can be either replication-defective or replication-competent. In most instances, replication-defective vectors, most commonly derived from adenovirus, herpesvirus, or retrovirus, also contain a gene for a toxin, cytokine, or antiangiogenic factor that can interfere with tumor growth or cause tumor regression. One approach involves the intratumoral introduction of a tumor suppressor, such as p53 or Rb, which can function to constrain tumor proliferation and, in certain cases, induce apoptosis. Another approach uses vectors to insert immune-stimulating or drug susceptibility “suicide” genes, such as the HSV thymidine kinase gene (HSV-\(tk\)) (Oldfield, Ram et al. 1993; Ram, Culver et al. 1993; Blaese, Ishii-Morita et al. 1994; Culver 1996; Blaese 1997; Touraine, Ishii-Morita et al. 1998; Tjuvajev, Chen et al. 1999; Wildner, Blaese et al. 1999). For certain types of tumors, a bystander-killing effect can be observed with a suicide gene such that only a percentage of the tumor cells needs to be transduced for eradication of a tumor. However, because they do not replicate, these vectors generally do not have significant antitumor effects on their own, but act as a vector for gene delivery.

Replication-competent vectors derived from viruses such as adenovirus or HSV can replicate intraneoplastically and are thus inherently cytotoxic to tumor cells. Moreover, recent studies have shown that the antitumor effect of these vectors following treatment of mouse models of cancer is more complex than direct cell killing, and may result from a combination of bystander killing effects and antitumor immune responses. The antitumor effects can be further enhanced by treatment with chemotherapy and radiation, which suggests that these therapies work synergistically and can be coadministered for treating a variety of human cancers.
1.18.2 Attenuation of HSV Vectors

Approximately 90% of the adult population has acquired antibodies to HSV due to previous exposure to the virus. Although neurovirulent in its wild-type form, a variety of mutations can be introduced that abrogate this toxicity. The attenuation of 'wild-type' laboratory strains of HSV increases the clinical benefit by reducing the virulence of the virus while retaining its tumor killing property. A breakthrough in this regard came with the discovery that HSV contains two copies of a gene called \( \gamma_{34.5} \) encoding a multifunctional protein that is essential for effective replication in certain cell types. It is basically a "neurovirulence factor", and its function is necessary for the virus to cause disease in animal models. One function of this protein is to counteract the effects of the natural antiviral response mounted by cells following infection, which involves activation of the cellular ds RNA-dependent protein kinase R (PKR). PKR activation leads to phosphorylation of the alpha subunit of the eukaryotic translation initiation factor, eIF2, which blocks translation and leads to cell death (Chou, Kern et al. 1990; He, Gross et al. 1998). However, this also effectively blocks the production of new virus particles. In essence, the cell commits suicide, which limits virus dissemination. The expression of virally-encoded \( \gamma_{34.5} \) protein following infection counteracts this cellular PKR response by preventing the cessation of protein synthesis long enough for the virus to replicate. Thus, the net effect of deleting the \( \gamma_{34.5} \) genes from the virus is to block replication in many cell types and to eliminate the ability of HSV to cause disease in animals. It was discovered, however, that many neoplastic cells retain the ability to support the replication of \( \gamma_{34.5} \) deficient HSV vectors, perhaps due to an altered PKR response or other genetic changes commonly found in cancer cells. The ability to kill tumor cells without causing disease in the host provides the rationale for developing replication-competent HSV vectors for cancer therapy.

1.18.3 Using HSV Vectors in Conjunction With Established Cancer Therapies

Because conditionally-replicating HSV vectors kill tumor cells through pathways that are different from other anticancer therapies, it is reasonable to explore the interactions with other commonly used antineoplastic agents. In one study of G207 (\( \gamma_{34.5} \)-negative HSV), intraneoplastic inoculation of head and neck squamous cell tumors
caused all tumors to regress initially, but tumors recurred in > 50% of animals. Likewise, cisplatin is a common agent used with variable success to treat this form of cancer. Because cisplatin at physiologically relevant doses does not appear to inhibit G207 replication in cultured cells, the combination of these therapeutics seems promising. Using a tumor cell line that is moderately sensitive to cisplatin, Chalavi and colleagues showed that a cure rate of < 15% with cisplatin alone or < 50% with G207 alone could be increased to 100% when cisplatin and G207 were used in combination (Chalavi, Todo et al. 1999).

Radiation therapy is also commonly used for the treatment of solid neoplasms. Investigations of the interactions of radiotherapy with HSV oncolytic vector therapy have only recently been reported. Studies of prostate cancer demonstrated that tumors recurring after irradiation do not develop resistance to HSV therapy (Walker, McGeagh et al. 1999). As with cisplatin treatment, irradiation does not appear to decrease viral efficacy, and additive or even synergistic effects are seen in appropriate circumstances (Advani 1998; Advani, Chung et al. 1999; Walker, McGeagh et al. 1999).

### 1.18.4 Disseminated Disease

The use of HSV-based oncolytic vectors has been successful for solid tumors. Unfortunately, these approaches do not necessarily treat secondary, distal tumors that are not directly accessible by the vector. To treat metastatic cancer, immunologic approaches have been attempted in which cytokines, co-stimulatory molecules, tumor-associated antigens, and/or major histocompatibility complex Class I molecules have been delivered to tumors in an attempt to induce a systemic, antitumor response. Many of these approaches have been shown to be effective in eradicating immunogenic tumors in murine models, and are now being used clinically.

Although there are many current gene therapy clinical protocols for cancer, the delivery of granulocyte-macrophage colony-stimulating factor to tumor cells ex vivo followed by intradermal injection for the genetically modified cells has resulted in a strong antitumor response in the treatment of melanoma and of some renal cell carcinoma (Dranoff, Jaffee et al. 1993; Dranoff, Soiffer et al. 1997). Granulocyte-macrophage colony-stimulating factor appears to induce dendritic cell maturation and results in
improved tumor antigen presentation. The $T_h1$ cytokine, IL-12, also appears to induce a strong antitumor response in mouse models when delivered directly to a solid tumor by injection of genetically modified fibroblasts. The results of a Phase 1 trial at the University of Pittsburgh have shown that the local production of IL-12 results in tumor regression, but whether there is an induction of systemic immunity must be determined. Still other applications of gene therapy to treating cancer include the delivery of drug resistance genes, such as dihydrofolate reductase and multidrug resistance gene-1, to bone marrow cells to increase their resistance to the toxic effects of chemotherapy.

1.18.4.1 Colorectal metastases to the liver

Metastatic colon carcinoma is second only to lung cancer as a cause of death from malignancy in the United States, accounting for 60,000 fatalities a year. Eighty percent of the patients who die of colon cancer have metastases in the liver (Fong, Blumgart et al. 1995). Currently, available treatments for patients with unresectable liver metastases offer essentially no hope for a cure (Millikan, Staren et al. 1997). The prognosis of patients with unresectable liver metastases derived from colorectal cancer is invariably poor. Metastatic disease from colorectal cancer, especially to the liver, is a major source of mortality. Radiotherapy and immunotherapy are not effective for secondary tumors. Chemotherapy has been the regimen of choice, although response to this therapy is often poor. Therefore a new systemic type of therapy is required.

1.19 Systemic Delivery

Recent evidence suggests that HSV vectors can effectively limit tumor growth when delivered by multiple routes, including intraperitoneal, intrapleural, intra-arterial, and intravenous. Although these experiments were carried out in rodents, these animals are susceptible to toxic effects of wild-type HSV and are reasonably good hosts for HSV replication. In this regard, demonstrations of efficacy in syngeneic models of disease show that HSV has an inherent ability to destroy tumors while sparing normal tissue at the doses tested.

Intravenous administration of vectors, while not providing tissue specificity, provides a simple means of administration and the potential for treating multiple tumor sites resulting from metastatic disease. Recent experiments with HSV delivered by the
hepatic artery or portal vein in rodents for the treatment of hepatoma or colon carcinoma metastatic to the liver show a profound reduction in tumor nodule formation (Kooby, Carew et al. 1999). In this manner, the same hypervascularity that the tumor generates for its growth could be exploited to increase regional viral delivery. More recently, HSV has been delivered intravenously into the tail vein of mice in an attempt to treat prostate tumors in a flank model of disease. In these experiments, intravenous delivery of G207 was shown to cause tumor regression, and 25% of the animals were cured (Walker, McGeagh et al. 1999). It is unlikely in these instances that a large proportion of the injected dose remains in the tumor because virus can be detected throughout the animal. It may be that a relatively small amount of virus is effective because it can establish a productive infection in tumor cells, and therefore generate a local toxic response. Moreover, the ability of viral vectors to elicit antitumor immunity may also come into play in this model. Additional experiments to sort out the mechanism of action in these models will be useful to resolve these issues.

1.19.1 Hemodynamics of Systemic Delivery

Blood-borne molecules or particles that enter the tumor vasculature reach cancer cells via distribution through the vascular compartment, transport across the microvascular wall, and transport through the interstitial compartment. For a molecule of given size, charge, and configuration, each of these transport processes may involve diffusion and convection. In addition, during the journey, the molecule may bind nonspecifically to proteins or other tissue components, bind specifically to the target(s), or be metabolized (Jain 1994).

1.19.1.1 Distribution through vascular space

The tumor vasculature consists of both vessels recruited from the pre-existing network of the host vasculature and vessels resulting from the angiogenic response of host vessels to cancer cells (Jain 1988; Folkman 1995). Movement of molecules through the vasculature is governed by the vascular morphology (i.e., the number, length, diameter, and geometric arrangement of various blood vessels) and the blood flow rate (Less, Skalak et al. 1991; Gazit, Berk et al. 1995; Baish, Gazit et al. 1996).
Although the tumor vasculature originates from the host vasculature, and the mechanisms of angiogenesis are similar (Folkman 1995; Patan, Munn et al. 1996), its organization may be completely different depending on the tumor type, its growth rate, and its location (Less, Skalak et al. 1991). The architecture and blood flow are different not only among various tumor types but also between a spontaneous tumor and its transplants (Jain 1988). For example, unlike normal tissue, where red blood cell (RBC) velocity is dependent on vessel diameter, there is no such dependence in tumors (Leunig, Yuan et al. 1992; Yuan, Salehi et al. 1994). Furthermore, RBC velocity may be an order of magnitude lower in tumors compared to the host vessels. The temporal and spatial heterogeneity in tumor blood flow may, in part, be a result of elevated geometric and viscous resistance in tumor vessels (Sevick and Jain 1989; Sevick and Jain 1991; Less, Posner et al. 1997), coupling between high vascular permeability and elevated interstitial fluid pressure (Netti, Roberge et al. 1996), and vascular remodeling (Patan, Munn et al. 1996).

Based on perfusion rates, four regions can be recognized in a tumor: an avascular, necrotic region, a semi-necrotic region, a stabilized microcirculation region, and an advancing front (Endrich, Reinhold et al. 1979) (Figure 1.7). Blood flow rates in necrotic and semi-necrotic regions of tumors are low, while those in non-necrotic regions are variable and can be substantially higher than in surrounding host normal tissues (Vaupel and Jain 1991). Considering these spatial and temporal heterogeneities in blood supply, coupled with variations in the vascular morphology at both microscopic and macroscopic levels, it is not surprising that the spatial distribution of nutrients and therapeutic agents in tumors is heterogeneous and that the average uptake decreases, in general, with an increase in tumor weight.
Figure 1. 7 Physiological barriers that a blood-borne molecule encounters before reaching a cancer cell in a solid tumor.

(A) Schematic of a heterogeneously perfused tumor showing well-vascularized periphery, a semi-necrotic, intermediate zone, and an avascular, necrotic central region. (B) Low interstitial pressure in the periphery permits adequate extravasation of fluid and macromolecules. (C) These macromolecules move toward the center by the slow process of diffusion. In addition, interstitial fluid oozing from the tumor carries macromolecules with it by convection into the normal tissue. Interstitial movement may be further retarded by binding. Products of metabolism may be cleared rapidly by blood.
1.19.1.2 Metabolic microenvironment

The temporal and spatial heterogeneities in blood flow are expected to lead to a compromised metabolic microenvironment in tumors. Both pH and pO₂ decrease as one moves away from tumor vessels, leading to acidic and hypoxic regions in tumors. While low pO₂ and pH are detrimental to some therapies (e.g. radiation), they might enhance the effect of certain drugs, if the drug could be delivered in adequate quantities in those regions (Jain, Shah et al. 1984; Nozue, Lee et al. 1996).

1.19.1.3 Transport across the microvascular wall

The transport of a molecule across normal or tumor vessels is governed by three transport parameters: (i) the surface area for exchange; (ii) transvascular concentration gradient; (iii) transvascular pressure gradient. Tumor vascular permeability is significantly higher than that of normal tissues (Dvorak, Brown et al. 1995). Hence these vessels may lack selectivity (Yuan, Dellian et al. 1995). Moreover, positively-charged molecules have been shown to have a higher permeability (Dellian, Yuan et al. 1996).

Not only does the vascular permeability vary from one tumor to the next, but within the same tumor it varies both spatially and temporally (Jain 1987). The local microenvironment plays an important role in controlling vascular permeability. Thus it is likely that the host-tumor interactions control the production and secretion of cytokines associated with permeability changes (e.g. vascular endothelial growth factor (VEGF) and its inhibitors). Although tumor vessels are indeed leaky to fluid and macromolecules, it is important to note that the high interstitial pressure created within the solid tumor limits the extravasation of agents into various regions of the tumor.

1.19.1.4 Transport through interstitial space

Once a molecule has extravasated, its movement through the interstitial space occurs by diffusion and convection (Jain 1987). As mentioned earlier, the interstitial fluid pressure is high in the center of tumors and low in the periphery and surrounding tissue. Therefore, one would expect interstitial fluid motion from the tumor's periphery into the surrounding normal tissue. In various animal and xenograft tumors studied to date, 6-14% of plasma entering the tumor has been found to leave from the tumor.
periphery (Jain 1987; Jain 1989). A macromolecule at the tumor periphery has to overcome this outward convection to diffuse into the tumor.

1.20 Hypotheses and Objectives

The research presented in the next set of chapters involves the investigation as to why biological therapies work or fail. From this, we can broaden our understanding of biological systems and the mechanism of action of therapies in these systems, and exploit this knowledge to further improve upon their safety and therapeutic index.

HSV vectors have a number of inherent advantages: (i) its large genome allows the vector to package a large payload; (ii) its genome exists as an episome in the host cell nucleus; (iii) seropositivity does not preclude repeated administration of the vector; (iv) it has a broad host range and can efficiently infect non-dividing cells. However, HSV is unable to infect mature skeletal muscle. This obstacle has hampered the development of HSV for muscle-directed gene therapy.

Therefore the rationale for this initial study was to investigate the basis for this age-dependent loss of myofiber transduction. HSV uses cell surface HS as initial attachment receptors. This interaction tethers the virus to the cell surface such that HSV can engage secondary protein receptors for viral entry. It was found that mature myofibers did not display a full complement of HS, and that this could be responsible for the block to HSV infection. Moreover, DS had been shown previously to mediate infection of cells normally refractory to HSV infection by acting as an artificial receptor for viral attachment. In this study, DS was able to rescue mature myofibers infection, thereby expanding the development of HSV vectors for muscle-directed gene therapy. Thus DS could be used to target infection of HSV to host cells that display altered GAGs.

Along these lines, cancer cells have been typically shown to express altered cell surface GAGs. Therefore we hypothesized that DS-coated virus would be able to target oncolytic virus to tumor in a systemic model of cancer. DS is normally an inhibitor of viral infection of host cells that have a full complement of cell surface GAGs. Since GAG expression is ubiquitous on most cell types, DS-coated virus would theoretically decrease viral accumulation in tissues and increase levels of tumor infection. In addition, DS embodies a number of properties that could be beneficial in a systemic delivery...
system. It has been shown to be a short-term volume expander, therefore enabling selective extravasation of viral particles to tumor due to the characteristic leakiness of tumor vasculature. Furthermore, it has been employed as a drug carrier to allow for protection and time-release of macromolecules in circulation. Despite the finding that DS inhibited infection of colorectal cancer cells, it significantly improved antitumor efficacy in both solid and metastatic tumor models.

This treatment was successful at delaying the growth of tumors and, in some instances completely eradicating them. Thus, it was important to uncover the biological basis for this therapy for a number of reasons. The implications of this involve not only an increased understanding of viral therapies and animal systems, but also an evaluation of the safety of treatments that may later be tailored for human use. Finally, investigations of modes of action for biological therapies enable researchers to exploit specific features in order to further improve their efficacy and safety.

With this in mind, we proceeded to investigate the properties of HSV, DS, and the animal models to determine how systemic delivery of relatively low doses of virus could result in marked antitumor efficacy. During the course of this investigation, we discovered that DS protected the virus in circulation and thereby increased its bioavailability. In addition, DS altered the hemodynamic properties of oncolytic HSV in circulation to allow for passive targeting of HSV to the tumor area. Once at the tumor site, extensive oncolysis was likely mediated primarily by endothelial cell apoptosis.

Based on this working model of systemically delivered HSV, we hypothesized that efforts to increase HSV infection of tumor vasculature should further improve the therapeutic index of our vector. By utilizing tumor-targeting peptides complexed with HSV, as well as antiangiogenic viruses, we found that antitumor efficacy was again significantly enhanced and survival considerably prolonged.

The set of hypotheses that outline the work presented in this thesis illustrate circular benefits to medical research: uncovering the biological basis for diseases lead to the development of novel therapies, which can then be further improved by investigating their mechanism of action. Consequently, we gain not only a greater understanding of animal biology, virology, and disease, but also an appreciation for how this knowledge can be applied to improving current standard protocols of health care.
CHAPTER 2: Materials and Methods

2.1 Materials

A complete list of chemical reagents and suppliers appears in Appendix 1.

2.2 Cells and Viruses

Vero cells were a gift from S. McKnight. The parental L cell used was the clone 1D line of Lmtk’ mouse fibroblast (Tufaro, Snider et al. 1987). The procedure for the isolation of the mutant sog9 cell line was described previously (Banfield, Leduc et al. 1995). Vero, L, and sog9 cells were grown at 37°C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO2 atmosphere. CT26 colon carcinoma cells were obtained from ATCC (Rockville, Maryland) and maintained in RPMI medium 1640 containing 10% FBS and L-glutamine.

Recombinant HSV-1, G207 (Medigene Inc.), R8102 (B. Roizman), L1BR1 (B. Roizman) (Nishiyama, Yamada et al. 1992; Asano, Honda et al. 1999), NV1020 (Medigene Inc.) (Meignier, Longnecker et al. 1988; Meignier 1991), NV1066 (Medigene Inc.), and angiostatin/endostatin viruses (J. Markert) were prepared on Vero cells. G207 contains the β-galactosidase gene inserted in-frame in the ribonucleotide reductase gene and both copies of γ34.5 deleted. As such, this recombinant virus possesses attenuated neurovirulence and is unable to replicate in non-dividing cells. L1BR1 is a recombinant HSV-2 that contains the β-galactosidase gene inserted into the US3 protein kinase gene. R8102, a mutant HSV-1 F strain that has the β-galactosidase gene inserted between UL3 and UL4, under the ICP27 promoter, was a gift from B. Roizman. R8102 displays LD50 values similar to wild type HSV-1 strain F (B. Roizman, personal communication).

NV1020 is a replication-competent HSV vector containing only one copy of γ34.5 and a deletion in the terminal repeats such that rearrangement of genome segments is ablated. NV1066 is a replication-competent HSV vector containing only one copy of γ34.5 and the green fluorescent protein (GFP) gene inserted at the terminal repeats. Both angiostatin and endostatin viruses are based on the G207 backbone, with the
antiangiogenic gene inserted in place of β-galactosidase. All viruses were propagated and titered on Vero cells.

2.3 **Viral Stock Production**

Subconfluent monolayers of Vero cells in 150 mm dishes were incubated with virus at a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU) per cell in DMEM. After 1 hour, cells were washed once with phosphate buffered saline (PBS) and overlaid with DMEM/10% FBS for 4 days. Cells and media were collected into a 50 ml conical tube, and centrifuged at 100 x g for 3 min. Supernatant was collected. The cell pellet was resuspended in 1 ml of PBS, which was then subjected to 3 freeze-thaw cycles in a dry ice/ethanol bath. The cell lysates were centrifuged at 250 x g for 10 min, and the virus-containing supernatants were pooled. The supernatant was filtered through a 0.45 μm filter. This was then layered onto a 3 ml solution of 30% sucrose in PBS in polycarbonate ultracentrifuge tubes, and centrifuged at 100 000 x g (Beckman SW 28 rotor) for 4 hours. The pellet was resuspended in 1 ml of 10% glycerol in PBS, distributed into aliquots, and stored at −80°C. Viral stocks were titered on Vero cells.

2.4 **Determination of Virus Titer**

To determine the titer of virus stocks, 1 x 10⁶ Vero cells were plated in 6-well dishes and infected 5 hours later with serial 10-fold dilutions of virus stock in DMEM. After a 1 hour adsorption period, the inoculum was removed and cell monolayers were washed three times with PBS to remove unbound virus. Cell monolayers were then overlaid with DMEM containing 4% FBS and 0.4% pooled human IgG. As the infection progresses, IgG neutralizes any extracellular virus, while intracellular virus spreads by direct cell-to-cell contact, thereby allowing a plaque to form. At 3-4 days post-infection, cell monolayers were washed with PBS and plaques were visualized by staining with 70% methanol/5% methylene blue for 30 min.

2.5 **Animal Care Protocol**

BALB/c mice were bred in institutional animal care facilities at the University of British Columbia. Animals were housed 5 per cage and provided food and water ad
libitum. Two different age groups were designated to be “newborn” and “adult”. The “newborn” mice were 7-10 days old. The “adult” mice were 6-12 weeks old. For experiments not detailing specific age groups, mice were approximately 20 g or 3-4 weeks old. Animal studies were performed in accordance with guidelines of the Animal Care and Use Committee of the University of British Columbia.

2.6 Primary Muscle Fiber Cultures

Single isolated myofibers were prepared from dissected extensor digitorum longus (EDL) muscle. The myofibers were dissociated by enzymatic disaggregation in 0.2% type 1 collagenase, followed by mild trituration. Isolated myofibers were then plated into several 24-well dishes coated with 1 mg/ml of Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). Culture medium consisting of 10% horse serum and 10% FBS in DMEM was added to the wells. These plates were then incubated for 18 hours at 37°C, at which point viable myofibers were subjected to viral infection.

2.7 Infection of Myofibers

Myofibers were infected by adding $10^6$ PFU of G207 in the culture medium (10% FBS/DMEM) directly to the wells. Incubation length was overnight (approximately 18 hours), although a 1 hour infection in DMEM only was sufficient to give reproducible infection. Following incubation, myofibers were fixed for 15 min in 1.25% glutaraldehyde and stained with 2% X-gal substrate (1 mM MgCl$_2$, 5mM K$_4$Fe(CN)$_6$/K$_3$Fe(CN)$_6$ in PBS) for 4 hours at 37°C.

2.8 In Vitro Myofiber Treatment Assays

Assays for dextran sulfate stimulation, collagenase type IV, chondroitin ABC lyase, and heparitinase were performed on adult myofibers plated in 24-well dishes. The myofibers were pretreated with varying concentrations of dextran sulfate, collagenase type IV, chondroitin ABC lyase, or heparitinase in DMEM for 30 min prior to infection. After an overnight adsorption period (approximately 18 h) at 37°C, the inoculum was removed. The myofibers were then fixed for 15 min in 1.25% glutaraldehyde and stained...
with 2% X-gal substrate for 4 h at 37°C. For all in vitro studies, a minimum of 40 myofibers was tested per treatment group unless otherwise specified.

PEG-induced fusion was performed according to methods described previously (Meyer, Hanon et al. 1998).

2.9 Anion Exchange Chromatography of Glycosaminoglycans

Biochemical labeling of glycosaminoglycans was performed by a modification of procedures described previously (Bame 1989). Briefly, glycosaminoglycans were radiolabeled by incubating cells for 24 h with \[^{35}\text{S}\]\text{sulfate} (carrier free, approximately 43 Ci/mg, ICN) per ml in DMEM/10% FBS/10% horse serum modified to contain 10 μM sulfate. The cells were washed three times with cold PBS and solubilized with 1 ml of 0.1 N NaOH at RT for 15 min. Samples were removed for protein determination. Extracts were adjusted to pH 5.5 by the addition of concentrated acetic acid and treated with protease (Sigma; 2 mg/ml) in 0.32 M NaCl-40 mM sodium acetate, pH 5.5, containing shark cartilage chondroitin sulfate (2 mg/ml) as carrier, at 40 °C for 12 h. For some experiments, portions of the radioactive material were treated for 12 h at 40 °C with 10 mU of chondroitin ABC lyase (Sigma) or 0.5 U of heparitinase (Sigma). The radioactive products were quantified by chromatography on DEAE-Sephacel (Pharmacia) by binding in 50 mM NaCl followed by elution with 1 M NaCl. For high pressure liquid chromatography (HPLC) analysis, the glycosaminoglycan samples were desalted by precipitation with ethanol. Following centrifugation, the ethanol precipitates were suspended in 20 mM Tris (pH 7.4) and resolved by anion-exchange HPLC, using TSK DEAE-35W column (15 by 75 mm; Beckman instruments). Proteoglycans were eluted from the column by using a linear 50 to 700 mM NaCl gradient formed in 10 mM KH₂PO₄ (pH 6.0). All buffers contained 0.2% Zwittergent 3-12 (Calbiochem). The glycosaminoglycans shown in HPLC profiles were identified by digestion of the sample with the relevant enzymes prior to chromatography.

2.10 Indirect Immunofluorescence Microscopy

Isolated myofibers were plated onto glass coverslips and infected with G207 as described in Section 2.7. They were then fixed in 1.25% glutaraldehyde in PBS for 15
min, rinsed twice with PBS, followed by 15 min incubation in the blocking solution (PBS with 1% bovine serum albumin). After blocking, myofibers were permeabilized with 0.1% Triton-X100/PBS for 5 min and incubated with a mouse anti-ICP4 antibody (B. Roizman) at 1:500 for 1 hour. Myofibers were washed with three changes of PBS, then incubated with goat anti-mouse IgG conjugated to Texas-Red (Jackson Immunochemicals) diluted 1:200 in blocking solution for 30 min. The myofibers were then rinsed with PBS and mounted on glass slides.

For cancer experiments, samples were dissected from animals and frozen in OCT over dry ice. Sections (10 μm) were obtained and dried at room temperature overnight. These were then fixed in cold acetone for 2 min, rinsed twice with PBS, followed by 30 min incubation in the blocking solution (PBS with 10% goat serum). After blocking, sections were incubated with mouse anti-rat CD31 antibody (BD Pharmingen) at 1:250 for 1 hour. Sections were washed with three changes of PBS, then incubated with goat anti-mouse IgG conjugated to Cy-5 (Jackson Immunochemicals) diluted 1:200 in blocking solution for 30 min. The sections were then rinsed with PBS and mounted with AquaPerm (Immunon).

Immunofluorescence staining was observed using a BioRad MRC 600 confocal epifluorescence microscope. Confocal images were rendered using NIH Image Version 1.60 and colorized with Adobe Photoshop Version 6.0 (Adobe Systems). Standard control experiments were performed, including incubation with the secondary antibody only and with mock infected cells. All fixation and antibody incubations were performed at room temperature.

### 2.11 Intramuscular Administration of Herpes Simplex Virus

Adult and newborn mice under anesthesia (Ketamine/Rhompun intraperitoneal injection) were injected percutaneously into the tibialis anterior muscle (TA) to an approximate depth of 2.0 mm using a Hamilton syringe. For in vivo assays involving co-injection of treatment solutions along with the viral inoculum, dextran sulfate, collagenase type IV, or chondroitin ABC lyase was diluted to the appropriate concentration (as identified by in vitro studies) with G207 in an injection volume of 50 μl (for adult mice) or 25 μl (for newborn mice). Control muscles were injected with G207.
only. To evaluate myofiber infection, muscles were removed 3 days post-injection for sectioning and histological analysis. For any of the procedures, a minimum of 5 animals received identical treatment and comprised an experimental group.

2.12 **RT-PCR**

Total cellular RNA was isolated from murine L, sog9, primary immature myofiber cultures, and primary mature myofiber cultures using TRIZOL Reagent, purified using oligo d(T) cellulose columns, reverse transcribed and then amplified using the following primer sets. EXT1 specific primers: 5' – CCG GAA TTC CGG AAG TCG TTC AAT GTC TCT G – 3' and 5' – CCG GAA TTC CGG AAG TCG CTC AAT GTC TCG GTA – 3'. EXT2 specific primers: 5' – AAG TAG CGC CGG GGA CCT GG – 3' and 5' – CGG GAT CCC GGT TTC ATT ATG TGT – 3'. Products were then analyzed by gel electrophoresis. Fragment size for EXT1 is 880 bp. Fragment size for EXT2 is approximately 800 bp.

2.13 **Tissue Sectioning**

The injected and control muscles were frozen in isopentane cooled in liquid nitrogen. Frozen muscles were sectioned, yielding serial cross-sections from one end of the muscle to the other. Cross-sections (10 μm) were cut on a cryostat and stained with X-gal and/or hematoxylin and eosin. The sections were retained at regular intervals (approximately every 120 μm). For histology, the cryosections were collected onto gelatin-coated glass slides.

Tumors were frozen in OCT over dry ice and sectioned, yielding serial cross-sections. Cross-sections (10 μm) were cut on a cryostat and stained with hematoxylin and eosin, or left unstained for immunohistochemistry (Section 2.10). The sections were retained at regular intervals (approximately every 120 μm). For histology, the cryosections were collected onto gelatin-coated glass slides.

2.14 **Histology**

The histological detection of β-galactosidase-expressing cells in cryosections was done using X-gal. This compound yields a blue reaction product in cells expressing high
levels of β-galactosidase. The sections were first fixed by dipping the slides in 4% paraformaldehyde in 100mM NaP, pH 7.2, for 5 min. The slides were rinsed three times for 5 min in PBS. The sections were then stained with X-gal (Sigma) at a concentration of 1 mg/ml in 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ in PBS for 12 hours. The slides were mounted using an aqueous mounting medium (Promount) and examined microscopically for the presence of β-galactosidase-labeled ("blue") myofibers. The total number of lacZ-expressing fibers in a muscle was determined from the section with the maximal number of blue fibers, and that was invariably at the site of implantation.

2.15 Isolation of Radiolabeled Virus

Subconfluent monolayers of Vero cells in 150 mm dishes were incubated with virus at a MOI of 0.1 PFU in DMEM. After 1 hour, cells were washed once with PBS and labeled for 48 hours with 25 μCi of [³⁵S]-methionine in methionine-free medium containing 5% dialyzed FBS and 10% complete DMEM. Cells and media were collected into a 50 ml conical tube, and centrifuged at 100 x g for 3 min. Supernatant was collected. The cell pellet was resuspended in 1 ml of PBS and then subjected to 3 freeze-thaw cycles in a dry ice/ethanol bath. The cell lysates were centrifuged at 250 x g for 10 min, and the virus-containing supernatants were pooled. The supernatant was filtered through a 0.45 μm filter. This was then layered onto a 3 ml solution of 30% sucrose in PBS in polycarbonate ultracentrifuge tubes, and centrifuged at 100 000 x g (Beckman SW 28 rotor) for 4 hours. The pellet was resuspended in 1 ml of 10% glycerol in PBS, distributed into aliquots, and stored at −80°C. Viral stocks were titered on Vero cells. Radiolabel incorporation was measured by liquid scintillation counting, and expressed as CPM/PFU.

2.16 Antibodies

Primary monoclonal antibodies directed against HSV ICP4 were obtained as a gift from B. Roizman. Monoclonal antibodies directed against rat CD 31 were obtained from BD Pharmingen.
Secondary antibodies used for immunofluorescence detection included goat anti-mouse IgG conjugated to Texas Red (Cedarlane) and goat anti-mouse IgG conjugated to Cy-5 (Jackson Immunoresearch).

2.17 Dextran Sulfate Assay for Cells

Assays for DS stimulation or inhibition were performed on sog9, L, and CT26 cells. The virus used for these experiments expresses β-galactosidase (G207). Confluent cell monolayers were pre-treated with DS in DMEM for 30 min prior to infection or inoculated with virus in DMEM previously mixed with DS. The cells pre-incubated with DS were rinsed three times with PBS prior to infection. After a 1 hour viral adsorption period at 37°C, the inoculum was removed and the cells were washed with PBS. The cells were then overlaid with culture medium (DMEM/10% FBS). After 10 hours, the cells were washed with three changes of PBS and stained with X-gal (Sigma) at a concentration of 1 mg/ml in 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ in PBS for 4 hours. Blue cells were visualized and counted.

2.18 One Step Growth Curve

Equal numbers of Vero and CT26 cells were plated onto 60 mm culture dishes. Twelve hours later, cells were infected at high (10) and low (0.1) MOI for one hour at 37°C in serum-free medium. Following infection, cells were rinsed three times with PBS, and culture medium was added to the dishes (DMEM/10% FBS with L-glutamine). Dishes were incubated at 37°C, 5% CO₂ for specified time points. At 2, 24, 48, and 72 hours, cells and supernatant were collected from dishes and stored at -80°C for titering. Experiments were performed in triplicate. PFU values reflect an average of 3 experiments.

2.19 Liver Metastatic Tumor Model

Animals were anesthetized using ketamine and xylazine, administered by intraperitoneal (i.p.) injection (70 mg/kg ketamine/10 mg/kg xylazine). Using the model described by Lafreniere and Rosenberg, 5 x 10⁴ syngeneic murine colon cancer cells (CT26), in 0.3 ml PBS, were injected into the exteriorized, inferior pole of the spleen.
through a 26-gauge needle (Lafreniere and Rosenberg 1986). The incision was closed and 24 h later, the wound was reopened and a single injection of 1) 300 µl of PBS (controls) or 300 µl of the following: 2) 1 x 10^7 PFU of NV1020; 3) 1 x 10^7 PFU of NV1020 plus 100 µg/ml DS; 4) DS alone (in PBS) was given to 6 animals per group. The animals were closed in a similar fashion to that described above and returned to their cages. Animal weights were followed closely (three times per week), and when control animals began to lose weight (approximately day 14 post tumor inoculation), animals were sacrificed by CO_2 inhalation, livers were harvested and tumor nodules counted.

2.20 Flank Tumor Model

Animals were anesthetized using ketamine and xylazine, administered by i.p. injection (70 mg/kg ketamine/10 mg/kg xylazine). 5 x 10^4 syngeneic murine colon carcinoma cells (CT26) in 100 µl of PBS were implanted into the right flank through a 26-gauge needle. Tumor volume is calculated according to the formula V = a x b^2 x 0.4, where a corresponds to the length and b corresponds to the width. When the established tumors reached a volume of 150 mm^3 (approximately 10 days), tail vein injections of the following treatments were administered to 8 animals per group every 2 days for 3 doses: a) 100 µl PBS (controls); b) DS alone (in PBS); c) 1 x 10^7 PFU of NV1020; d) 1 x 10^7 PFU of NV1020 plus 100 µg/ml DS. The animals were closely monitored and tumor volumes calculated every 2 days. When tumor volume attained 2500 mm^3, animals were killed by CO_2 narcosis. Efficacy and survival plots were drawn accordingly.

2.21 Pharmacokinetic Assay

BALB/c mice (18-22 g) were given a single lateral tail vein injection (100 µl) of the following doses: a) PBS only; b) 1 x 10^7 PFU of NV1020; c) 1 x 10^7 PFU of NV1020 plus 100 µg/ml DS. At various times post-injection, mice (4 per group) were terminated by cervical dislocation. Blood was immediately removed by cardiac puncture and collected into microfuge tubes on ice. Serum was then removed and tested for PFU by serial dilution titer assay.
2.22 **Biodistribution Assay**

Equivalent numbers of Vero cells were plated on 96-well dishes. An aliquot of serum was serially diluted to $10^{-7}$ and applied to respective wells in a 50 µl volume of growth medium. Three days post-infection, plates were stained with 10% methylene blue in methanol and dried. PFU was calculated according to reciprocal dilution required to see no clearing of Vero cells.

2.23 **Statistical Analysis**

P-values were calculated by Microsoft Excel according to the student’s T-test. In the instances that greater than 2 groups were being compared, the ANOVA and Kruskall-Wallis rank tests were conducted to determine the significance between treatment groups.

2.24 **Morris Hepatoma Model**

Animals were anesthetized using ketamine and xylazine, administered by i.p. injection (70 mg/kg ketamine/10 mg/kg xylazine). Using the model described by Lafreniere and Rosenberg, $5 \times 10^4$ syngeneic murine colon cancer cells (CT26), in 0.3 ml PBS, were injected into the exteriorized, inferior pole of the spleen through a 26-gauge needle (Lafreniere and Rosenberg 1986). The incision was closed and 3 weeks later, the wound was reopened and a single injection of 1) 300 µl of PBS with DS (controls) or 300 µl of the following: 2) $1 \times 10^8$ PFU of NV1066; 3) $1 \times 10^7$ PFU of NV1066 plus 100 µg/ml DS was given to 6 animals per group. The animals were closed in a similar fashion to that described above and returned to their cages. After 48 hours, animals were sacrificed by CO$_2$ inhalation, livers were harvested and frozen in OCT for sectioning and immunohistochemistry.
CHAPTER 3: Dextran Sulfate Can Act as an Artificial Receptor to Mediate Infection of Mature Skeletal Muscle

3.1 Introduction

Skeletal muscle is an ideal seeding site for the treatment of primary myopathies or diseases requiring production of circulating proteins because it is highly vascular and an excellent secretory organ with many accessible sites (Blau and Springer 1995; Isaka, Brees et al. 1996; Bohl and Heard 1997; Howell, Lochmuller et al. 1998; Pauly, Johns et al. 1998; van Deutekom, Hoffman et al. 1998) (Takeda 1997; Tsurumi, Kearney et al. 1997). Moreover, the post-mitotic nature and longevity of muscle fibers permits stable expression of transferred genes, even if they are not integrated into chromosomal DNA (Svensson, Tripathy et al. 1996; van Deutekom, Floyd et al. 1998). High level gene expression in a relatively small number of muscle fibers may be adequate to treat inherited or acquired metabolic disorders, or to induce an immune response sufficient for vaccination (Davis, Michel et al. 1993).

Gene transfer to skeletal muscles has been hampered in part due to the inability of current generation vectors to infect a significant number of cells (Acsadi, Dickson et al. 1991; Karpati and Acsadi 1993; Mulligan 1993; Smith, Mehaffey et al. 1993; Acsadi, Jani et al. 1994; Yang, Nunes et al. 1994; Dai, Schwarz et al. 1995; Huard, Lochmuller et al. 1995). Although AAV efficiently infects muscle and elicits sustained gene expression, its capacity for delivering and regulating large genes is limited. As for the large DNA viruses such as HSV and adenovirus, muscle fibers exhibit a maturation-dependent loss of susceptibility to infection (Quantin, Perricaudet et al. 1992; Ragot, Vincent et al. 1993; Vincent, Ragot et al. 1993; Acsadi, Jani et al. 1994; Huard, Goins et al. 1995; Huard, Feero et al. 1996; Inui, Okada et al. 1996; Feero, Rosenblatt et al. 1997; Huard, Akkaraju et al. 1997; Huard, Krisky et al. 1997). Previous studies of HSV infection in the rodent show that the loss of infectivity may be due, at least in part, to the development of the basal lamina throughout the course of maturation, which may block the initial events in HSV infection (Huard, Feero et al. 1996). To initiate infection, HSV attaches to cell surface glycosaminoglycans such as heparan sulfate and dermatan sulfate.
(Fuller and Lee 1992; Shieh, WuDunn et al. 1992; Spear, Shieh et al. 1992; Gruenheid, Gatzke et al. 1993; Herold, Visalli et al. 1994; Banfield, Leduc et al. 1995; Williams and Straus 1997), which stabilize the virus such that it can interact with secondary protein receptors required for entry into host cells (Montgomery, Warner et al. 1996; Geraghty, Krummernacher et al. 1998; Terry-Allison, Montgomery et al. 1998). Thus, one possibility to account for the loss of susceptibility to HSV infection is that maturation leads to a decrease in the expression of cell surface molecules required for efficient infection.

In this study, we demonstrate that glycosaminoglycan synthesis is downregulated during murine skeletal muscle maturation. This could account for the loss of HSV infectivity because heparan sulfate acts as a co-receptor for attachment of HSV to cells (Montgomery, Warner et al. 1996; Whitbeck, Peng et al. 1997; Geraghty, Krummernacher et al. 1998). To test whether secondary HSV receptors were present, myofibers were treated with a variety of enzymes, including collagenase type IV and chondroitin ABC lyase. Both of these treatments enhanced HSV infection, which suggests that virus receptors were present but not readily accessible to the virus in the intact myofiber. Surprisingly, we also found that infectivity of HSV-1, but not HSV-2, could be restored by exposing myofibers to low concentrations of the glycosaminoglycan analog dextran sulfate. Dextran sulfate has been shown previously to promote HSV-1 but not HSV-2 infection in the absence of heparan sulfate. This supports the hypothesis that a lack of accessible heparan sulfate is responsible for the resistance of mature myofibers to HSV-1 infection. Taken together, these results show that the basal lamina is not an absolute block to infection, and that dextran sulfate can be used as a surrogate co-receptor for the nondestructive targeting of HSV-1 to mature skeletal muscle. These findings greatly expand the usefulness of HSV as a gene therapy vector for the treatment of inherited and acquired diseases.
3.2 Results

3.2.1 Characterization of immature and mature myofiber susceptibility to HSV

It has been shown previously that HSV vectors infect newborn muscle fibers in vitro, but not those isolated from older animals (Huard, Feero et al. 1996; Feero, Rosenblatt et al. 1997; Huard, Akkaraju et al. 1997; Huard, Krisky et al. 1997). To investigate the underlying basis for the maturation-dependent loss of infection, single muscle fibers were established in culture, and exposed to G207, which is an attenuated replication-defective HSV-1 vector that expresses β-galactosidase following infection (Mineta, Rabkin et al. 1995; Yazaki, Manz et al. 1995). In these assays, newborn myofibers were completely susceptible to infection, whereas only 6% of mature myofibers were infected at this concentration of virus (Table 3.1 and Figure 3.1, a and b). Thus, these results were consistent with previous studies showing a maturation-dependent loss of susceptibility to HSV infection (Huard, Feero et al. 1996; Feero, Rosenblatt et al. 1997).
Table 3.1 Number of myofibers isolated from mature mouse EDL muscle that express lacZ following inoculation with G207 and proposed treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Number of Fibers</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>G207 only</td>
<td>88</td>
<td>6</td>
</tr>
<tr>
<td>cytosine arabinoside</td>
<td>41</td>
<td>5</td>
</tr>
<tr>
<td>cytosine arabinoside and 10 μg/ml dextran sulfate</td>
<td>49</td>
<td>100</td>
</tr>
<tr>
<td>0.02 mg/ml collagenase type IV</td>
<td>68</td>
<td>7</td>
</tr>
<tr>
<td>0.20 mg/ml collagenase type IV</td>
<td>81</td>
<td>31</td>
</tr>
<tr>
<td>0.33 mg/ml collagenase type IV</td>
<td>77</td>
<td>97</td>
</tr>
<tr>
<td>0.66 mg/ml collagenase type IV</td>
<td>67</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.3 μg/ml dextran sulfate</td>
<td>78</td>
<td>6</td>
</tr>
<tr>
<td>3.0 μg/ml dextran sulfate</td>
<td>75</td>
<td>7</td>
</tr>
<tr>
<td>10 μg/ml dextran sulfate</td>
<td>82</td>
<td>99</td>
</tr>
<tr>
<td>2 U/ml chondroitin ABC lyase</td>
<td>49</td>
<td>100</td>
</tr>
<tr>
<td>4 U/ml chondroitin ABC lyase</td>
<td>44</td>
<td>30</td>
</tr>
<tr>
<td>6 U/ml chondroitin ABC lyase</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>1 – 6 U/ml heparitinase</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>PEG</td>
<td>33</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.66 mg/ml collagenase type IV was toxic to isolated myofibers.
Figure 3.1 HSV ICP4 immunofluorescence showing infected nuclei of mature myofibers.

Isolated myofibers infected with G207 were processed for indirect immunofluorescence using a mouse anti-ICP4 antibody. (a) G207 only, mature myofiber; (b) G207 only, immature myofiber; (c) G207 only; (d) G207 + 0.33 mg/ml collagenase type IV; (e) G207 + 3 μg/ml dextran sulfate; (f) G207 + 10 μg/ml dextran sulfate; (g) G207 + 2 U/ml chondroitin ABC lyase; (h) G207 + 4 U/ml chondroitin ABC lyase. c-h, mature myofibers. Magnification: a, b, c, e-g x 20; h x 40; d x 60. Images (panel c-g) were captured by confocal microscopy and colorized by Adobe Photoshop (thereby green in color). Panels a and b represent immunofluorescence microscopy (not confocal), and thereby are red in color. Successful infection is indicated by individual nuclei lighting up. The nuclei lighting up in panel a illustrate myoblast infection, not myofiber infection.
3.2.2 Approaches to rescue adult myofiber infectivity

Previous studies have suggested that basal lamina formation during maturation may act as a physical barrier to HSV infection, thereby preventing interaction of the virus with the receptors required for entry. To test this, isolated myofibers were exposed to G207 following treatment with collagenase type IV, which liberates peptides from collagen thereby degrading the basal lamina (Table 3.1). Indirect immunofluorescence of a nuclear HSV protein, ICP4, revealed that partial destruction of the basal lamina in this manner stimulated HSV infection (Figure 3.1, d). The effect was concentration-dependent such that an increase in collagenase type IV correlated with an increase in HSV infection. Toxicity occurred at 0.66 mg/ml as indicated by myofiber hypercontraction during the 30 min preincubation period (Table 3.1). In a second approach, chondroitin ABC lyase, which degrades a broad range of chondroitin sulfate moieties, was tested for its ability to enhance susceptibility to HSV infection (Figure 3.1, g and h). This treatment strongly enhanced infection, whereas treatment with heparitinase did not (Table 3.1). Thus, partial destruction of the basal lamina with specific enzymes allowed for the attachment and entry of HSV into the mature muscle fiber, which suggests that virus secondary receptors were present but not accessible in the context of the mature myofiber.

3.2.3 Analysis of cell surface glycosaminoglycans

HSV infects cells by attaching to cell surface heparan sulfate-like moieties followed by interaction with secondary protein receptors (Spear, Shieh et al. 1992; Gruenheid, Gatzke et al. 1993; Montgomery, Warner et al. 1996; Geraghty, Krummernacher et al. 1998). Although not strictly required, cell surface heparan sulfate increases the efficiency of HSV infection by two orders-of-magnitude in most cells tested (Banfield, Leduc et al. 1995). To investigate whether glycosaminoglycan expression was altered in adult versus newborn muscle fibers, radiolabeled glycosaminoglycans were isolated from muscle fiber cultures and analyzed by anion-exchange HPLC (Figure 3.2). Newborn muscle fibers expressed significant amounts of heparan sulfate and chondroitin sulfate glycosaminoglycans. By contrast, glycosaminoglycan synthesis was significantly reduced in adult myofibers during steady-state labeling, and the residual heparan sulfate
synthesized was relatively under-sulfated when compared with newborn myofibers (Figure 3.2).
Figure 3. 2 Anion-exchange HPLC of cell-associated glycosaminoglycans derived from myofibers belonging to different age-groups.

Myofibers were labeled with $[^{35}\text{S}]$-sulfate for 24 h. The medium was removed, and the monolayers were washed extensively to remove any traces of medium from the cells. Glycosaminoglycans were isolated and fractionated by HPLC. HS, elution position of heparan sulfate; CS, elution position of chondroitin sulfate; open diamonds, myofibers isolated from 8 day old mice; closed diamonds, myofibers isolated from 2 month old mice. The dotted line represents the salt gradient used for elution.
3.2.4 Dextran sulfate restores HSV infection in mature myofibers

The data so far indicated that one or more components of the basal lamina present in mature myofibers inhibited HSV infection. Moreover, HS biosynthesis of mature myofibers was reduced when compared with immature myofibers, which could account for all or part of the loss of susceptibility to HSV infection. It has been shown previously that cells devoid of HS biosynthesis can be infected with HSV-1, but not HSV-2, if a low concentration of dextran sulfate is added to the cells either prior to or during infection (Dyer, Banfield et al. 1997). By contrast, DS is a potent inhibitor of HSV infection if the target cells express significant amounts of HS. When DS was added to mature myofibers in culture, HSV-1 infection was significantly enhanced (Figure 3.1, e and f, and Table 3.1). Infection was not mediated by myoblast fusion, since treatment with cytosine arabinoside did not reduce levels of infection (Table 3.1).

Moreover, this effect was specific for HSV-1, which is consistent with the hypothesis that the lack of mature myofiber infection was due, at least in part, to a lack of accessible heparan sulfate moieties on the cell surface (Table 3.2). It has been previously shown that DS is able to stimulate infection of HSV-1 but not HSV-2 in cells lacking cell surface HS (Dyer, Banfield et al. 1997). To determine whether the same type-specific interaction occurred in a skeletal muscle model, we treated mature myofibers with DS prior to challenge with HSV-1 or HSV-2. Interestingly, mature myofibers infection by HSV-1 (G207) but not HSV-2 (L1BR1) could be enhanced by the addition of DS (Table 3.2).

To test whether there was an additional block in the post-attachment fusion of HSV with the plasma membrane, isolated mature myofibers were exposed to the fusogenic agent PEG prior to challenge with G207. PEG-induced fusion did not alter adult myofiber infectivity, suggesting that the block to HSV infection occurred at the level of viral attachment (Table 3.1).

3.2.5 In vivo analysis of HSV and DS injection into mature skeletal muscle

To establish that immature myofibers were susceptible to G207 infection in vivo, 10^6 PFU were injected directly into the tibialis anterior muscle of an 8 day old BALB/c
mouse. Injected muscles were removed three days post-injection, sectioned, and analyzed histochemically for the expression of β-galactosidase (Figure 3.3). High levels of transgene expression were detected in the injected area. HSV-infected myofibers were also found away from the site of injection, which suggests that there was considerable spread of the vector.
Table 3.2 Dextran sulfate stimulation of mature myofibers with G207 (HSV-1) versus L1BR1 (HSV-2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus</th>
<th>β-galactosidase expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>G207</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>L1BR1</td>
<td>-</td>
</tr>
<tr>
<td>10 µg/ml dextran sulfate</td>
<td>G207</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>L1BR1</td>
<td>-</td>
</tr>
</tbody>
</table>

"-" indicates no β-galactosidase expression in any mature myofibers with stated treatment. Therefore HSV was unable to gain entry into these cells. "+" indicates β-galactosidase expression in mature myofibers with stated treatment. Positive cells were scored as blue cells following X-gal staining. In this case, HSV was able to gain entry into mature myofibers at levels within 5% as outlined in Table 3.1. Total number of fibers per treatment is 40. Immature myofibers were fully susceptible to HSV-1 and HSV-2 infection.
Figure 3.3 In vivo injection of immature skeletal muscle with G207.

Cryostat sections of immature mouse TA muscle taken 3 days after injection of HSV and stained histochemically for β-galactosidase. Gene transfer was carried out by intramuscular injection of G207 (1 x 10⁶ PFU) in a volume of 50 μl. a and b, G207 only. Magnification: a x 10; b x 40
To test whether the three treatments that enhanced infection *in vitro* worked in the adult animal, mice were injected with $1 \times 10^6$ PFU of G207 in the tibialis anterior muscle along with either chondroitin ABC lyase, collagenase type IV, or dextran sulfate. In all instances, the *in vivo* results were consistent with the observations made *in vitro* (Figure 3.4, Table 3.3). Interestingly, dextran sulfate could be administered an hour prior to virus with no loss of function, an observation also made *in vitro* (Dyer, Banfield et al. 1997). In addition, infection was not limited to regenerating myofibers, which were identified by their centrally-located nuclei. Taken together, these results show that the barrier to HSV infection in adult skeletal muscle was due, at least in part, to the relative paucity of HSV receptors required for efficient infection.
**Figure 3.4 Injection of mature skeletal muscle with G207.**

β-galactosidase gene transfer to skeletal muscle of adult mice. G207 (1 x 10^6 PFU) and the proposed treatments were co-injected into the tibialis anterior of 2 month old BALB/c mice in a total injection volume of 50 μl. Frozen sections were cut (cross-sections) and stained for β-galactosidase activity. *a* and *b*, G207 only; *c* and *d*, G207 + 0.33 mg/ml collagenase type IV; *e* and *f*, G207 + 10 μg/ml dextran sulfate; *g* and *h*, G207 + 2 U/ml chondroitin ABC lyase. *a* and *b*, interstitial staining only. Magnification: *a*, *c*, *e*, *g* x 20; *b*, *d*, *f*, *h* x 40.
Table 3.3 Number of lacZ-expressing fibers in mature mouse TA muscle following gene transfer by intramuscular co-injection of treatment with G207

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Number of blue fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>G207 only</td>
<td>0</td>
</tr>
<tr>
<td>3 μg/ml dextran sulfate</td>
<td>76</td>
</tr>
<tr>
<td>10 μg/ml dextran sulfate</td>
<td>149</td>
</tr>
<tr>
<td>0.18 mg/ml collagenase type IV</td>
<td>0</td>
</tr>
<tr>
<td>0.33 mg/ml collagenase type IV</td>
<td>77</td>
</tr>
<tr>
<td>2 U/ml chondroitin ABC lyase</td>
<td>302</td>
</tr>
<tr>
<td>4 U/ml chondroitin ABC lyase</td>
<td>226</td>
</tr>
</tbody>
</table>

Sections from 4 animals per treatment group were used to calculate the average number of blue fibers. Values did not exceed an error margin of 10%.
3.2.6  **EXT1 is downregulated during skeletal muscle maturation**

Mutations in members of the exostosin (EXT) gene family have been implicated in hereditary multiple exostoses, a dominantly inherited genetic disorder characterized by multiple cartilaginous tumors. Recent studies have shown that both EXT1 and EXT2 harbor GlcA transferase and GlcNAc transferase activities that could catalyze the polymerization of HS (Lind, Tufaro et al. 1998). The HPLC profile of mature myofibers indicated that they did not display a full complement of GAGs, as compared to immature myofibers (Figure 3.2). Therefore to investigate whether the expression of EXT1 or EXT2 was altered during maturation, RT-PCR was performed with RNA isolated from newborn and adult myofibers cultures (Figure 3.5). EXT1 was not expressed in adult myofibers. This may account for the decrease in cell surface HS observed by HPLC.
Figure 3.5 EXT1 and EXT2 expression in immature and mature myofibers.

RT-PCR was conducted on the following cultures: L cell, sog9, immature myofibers, and mature myofibers. L cells display cell surface HS, whereas sog9 cells do not. Immature myofibers also display cell surface HS, although mature myofibers do not. Both enzymes are required for HS biosynthesis.
3.3 Discussion

The use of muscle as an ectopic site for the production of recombinant proteins has applications for many diseases (Isaka, Brees et al. 1996; Svensson, Tripathy et al. 1996; Bohl and Heard 1997; Takeda 1997; Tsurumi, Kearney et al. 1997). Expression of therapeutic proteins in even a small number of myofibers may be adequate to treat inherited or acquired metabolic disorders, or to induce an immune response in the context of a vaccine (Danko and Wolff 1994; Davis, Michel et al. 1995). Effective methods for muscle gene transfer require vector systems that transduce cells with high efficiency and allow for persistent transgene expression. However, viral vectors such as HSV and adenovirus possess limited ability to infect mature skeletal muscle.

We have shown that efficient infection of mature, highly differentiated myofibers by HSV vectors depends, at least in part, on the display of appropriate receptors on the cell surface. HSV, like several other pathogens, attaches to the host cell via heparan sulfate moieties displayed on the cell surface (Shieh, WuDunn et al. 1992; Spear, Shieh et al. 1992). Interactions with secondary protein receptors lead to fusion with the plasma membrane and a productive infection (Geraghty, Krummernacher et al. 1998). Our results indicate that maturation-dependent changes in heparan sulfate on the myofiber cell surface reduce the ability of HSV to infect these cells. This conclusion is supported by the demonstration that soluble dextran sulfate stimulates HSV infection. Heparan sulfate proteoglycans interact with a wide variety of ligands, including growth factors, cytokines, and other regulatory proteins, and it is not surprising to find an alteration in the expression of these moieties during differentiation and maturation. Previous studies have shown that skeletal muscle differentiation is accompanied by a downregulation of perlecan, a proteoglycan involved in the activation of tyrosine kinase receptors by basic fibroblast growth factor (bFGF) (Larrain, Alvarez et al. 1997; Larrain, Cizmeci-Smith et al. 1997). However, perlecan expression appears to remain stable during maturation (Huard, Feero et al. 1996). More recently, it has been shown that several GlcNAc N-deacetylase/N-sulfotransferase (NDST) genes are expressed poorly or not at all in mature skeletal muscle (Aikawa and Esko 1999). Our studies have also shown EXT1 downregulation in mature myofibers. These genes encode isozymes that catalyze crucial
reactions in the HS biosynthesis pathways that lead ultimately to the formation of specific oligosaccharide sequences with specific ligand binding properties. The loss or modification of these and potentially other HS biosynthetic activities could account, at least in part, for the loss of susceptibility to HSV infection that we observed.

AAV has also been shown to use heparan sulfate as a co-receptor to gain entry to cells (Summerford and Samulski 1998; Qing, Mah et al. 1999), and is capable of infecting mature myofibers quite efficiently (Clark, Sferra et al. 1997; Fisher, Jooss et al. 1997; Monahan, Samulski et al. 1998; Rendahl, Leff et al. 1998). There are several possibilities to account for this difference. The relatively small size of AAV compared with HSV (20 nm versus 200 nm) may allow it to better penetrate the basal lamina to engage its cognate receptors. Alternatively, it may not have as stringent a requirement for heparan sulfate moieties as does HSV to infect cells efficiently. Additional studies of the precise requirements for AAV versus HSV will be required to understand the differences in muscle susceptibility for these two viruses.

More importantly, however, our results show that the basal lamina is not an absolute block to HSV infection. Dextran sulfate, a highly sulfated glycosaminoglycan analog, was capable of stimulating HSV-1 infection, suggesting that mature myofibers can be made susceptible to this virus without destroying any component of the cell architecture. It has been shown previously that dextran sulfate stimulates HSV-1, but not HSV-2, infection of cells deficient in glycosaminoglycan biosynthesis (Dyer, Banfield et al. 1997), which is interesting because dextran sulfate is normally a potent inhibitor of enveloped virus infection (Banfield, Leduc et al. 1995; Banfield, Leduc et al. 1995; Dyer, Banfield et al. 1997). It appears that in the absence of heparan sulfate, dextran sulfate is able to bind to the virus and to the host cell, thereby tethering the virus to the cell surface. This allows the virus to establish multivalent contacts with additional protein receptors required for a productive infection. HSV-2 has been shown to lack this ability (Dyer, Banfield et al. 1997), and our results in myofibers are consistent in this regard. Thus, mature myofibers behave, in vitro and in vivo, as if they are relatively deficient in cell surface heparan sulfate, a result confirmed by HPLC analysis. Moreover, enzymatic digestion of chondroitin sulfate moieties or collagen type IV may stimulate infection (Table 1 and Figure 1) by exposing an underlying receptor, or by removing an inhibitory
molecule. Dextran sulfate obviates the need to remove these moieties, which shows that HSV receptors are accessible without destroying the mature myofiber.

 Previous studies have shown that gene delivery to muscle using viral vectors can elicit marked inflammatory responses that lead to a significant decline in the number of transduced myofibers. This lack of persistence of transgene expression is likely due to the elimination of transduced myofibers by the host immune response: cytotoxic CD4+ and CD8+ T cells and activated macrophages (Acsadi, Lochmueller et al. 1996; Huard, Akkaraju et al. 1997; Huard, Krisky et al. 1997). Adequate immunosuppression may be necessary to overcome this limitation of viral gene transfer. In fact, administration of immunosuppressive agents such as CTLA4Ig and FK506 have been shown to sustain transgene expression after adenovirus injection in skeletal muscle (Vilquin, Guerette et al. 1995; Guerette, Vilquin et al. 1996). However, preliminary studies with HSV amplicons have yielded successful results with our treatments. The use of non-replicating, relatively non-toxic HSV amplicons with our proposed treatments could further alleviate the immunogenicity of foreign protein expression in skeletal muscle.

 We have also shown in vitro, that infected myoblasts remaining in the culture are not responsible for the efficient myofiber transduction that we observe. Treatment of the myofiber culture with cytosine arabinoside kills myoblasts (actively dividing cells), but does not abrogate infection with dextran sulfate. Moreover, HSV-2 infects myoblasts and immature myofibers in culture quite efficiently, and yet does not infect mature myofibers. In vivo, the use of collagenase IV does in fact result in gross muscle damage, as indicated by hematoma formation in the injection area. Although this may indicate that myoblast-mediated infection of myofibers may be possible, one would expect increased myofiber transduction with increased collagenase IV concentration. This does not occur. In fact, none of the proposed treatments yield higher transduction efficiency with higher concentrations of treatment. Moreover, dextran sulfate and chondroitin ABC lyase appear to be relatively non-toxic following muscle administration. Dextran sulfate is currently being used for treatment of cardiovascular disease (arteriosclerosis) as well as being considered a possible treatment for AIDS.

 The ability of dextran sulfate to stimulate HSV-1 infection of mature muscle tissue is important because it is non-destructive, non-toxic, and it limits the spread of the
vector from disseminating to other sites. Therefore, this represents an approach for targeted expression of HSV vectors to muscle fibers by direct injection. HSV is attractive as a gene delivery vector because its large size allows for the delivery of several large genes at once, and it can be made relatively non-toxic (Glorioso, DeLuca et al. 1995; Huard, Krisky et al. 1997). Moreover, HSV can be grown to high titers, can infect nondividing cells efficiently (Lim, Hartley et al. 1996), and can be controlled through the action of antiviral drugs, such as acyclovir, that inactivate virus replication (Hasegawa, Emi et al. 1993; Black, Newcomb et al. 1996; Evrard, Vian et al. 1996). This study shows that HSV can efficiently transfer genes to mature muscle cells in vitro and in vivo, and lays the groundwork for the expanded use of HSV as a gene delivery vector. Moreover, elucidation of the biological basis for the critical age-dependent mechanism that restricts infectivity of mature skeletal muscle tissue provides basic insight into muscle cell biology and development, with potentially important therapeutic implications.
CHAPTER 4: Dextran Sulfate Enhances the Systemic Delivery of Oncolytic Herpes Simplex Virus For Treatment of Colorectal Cancer

4.1 Introduction

Colorectal cancer is the second leading cause of cancer deaths in the United States (Greenlee, Murray et al. 2000). Colorectal malignancies most frequently metastasize to the liver, and if these cases are left untreated, patients have a median survival of 5 to 10 months (Weiss, Grundmann et al. 1986; de Brauw and van de Velde 1987). Surgical resection may result in cures, however two-thirds of patients undergoing resection succumb to recurrence from residual microscopic disease (Panis, Ribeiro et al. 1992; Fong, Cohen et al. 1997). Despite moderate clinical responses from palliative chemotherapy, there is no curative treatment for unresectable metastases (Fong, Kemeny et al. 1996; Kemeny, Huang et al. 1999). Therefore novel agents are being developed to treat this disease.

Oncolytic viruses are suitable for treating individual tumors that are surgically accessible, and may be suitable for treating disseminated disease. As such, investigations into systemic modes of delivery are also being conducted. Recent evidence suggests that HSV vectors can effectively limit tumor growth in animal models of cancer when delivered by multiple routes, including intraperitoneal, intrapleural, portal, and intraarterial (Rainov, Dobberstein et al. 1998; Coukos, Makrigiannakis et al. 1999; Kasuya, Nishiyama et al. 1999; Bennett, Kooby et al. 2000; Yoon, Nakamura et al. 2000). In this regard, demonstrations of efficacy in syngeneic models of disease show that HSV destroys tumors while sparing normal tissue (Jia, McDermott et al. 1994; Mineta, Rabkin et al. 1995; Yazaki, Manz et al. 1995; Miyatake, Martuza et al. 1997).

The HSV vector described in this study (NV1020) is a replicating oncolytic HSV vector that was initially designed as a vaccine for HSV and is now currently in clinical trials for human colorectal liver metastases. NV1020 is a recombinant HSV-1 (strain F) containing one copy of the γ34.5 gene and a deletion in the internal inverted repeats. Other HSV vectors are being evaluated in the clinic for human malignant glioma.
HSV is a human neurotrophic virus with the ability to replicate in a wide variety of cells in culture, including tumor cells. Recent studies have shown that the primary receptors for HSV entry broadly expressed in human cells belong to an immunoglobulin subfamily. Nectin 1δ, also named Prr1 or HveC, and nectin 1α, also designated HIgR, mediate entry of all HSV-1 strains tested by binding to HSV glycoprotein gD. Interestingly, HSV uses heparan sulfate as an initial receptor prior to interaction with a secondary protein receptor for stable attachment. Heparan sulfate is widely expressed on the surface of human tissues and is frequently altered in cancer cells (Robinson, Viti et al. 1984; Kure and Yoshie 1986; Steck, Cheong et al. 1987; Iozzo 1988; Steck, Moser et al. 1989; Bouziges, Simon-Assmann et al. 1990; Nackaerts, Verbeken et al. 1997; Jayson, Lyon et al. 1998; Conejo, Kleeff et al. 2000).

DS is a GAG analog that is currently used clinically for treatment of vascular disease, including plasma volume expansion, flow improvement, and antithrombotic uses (Derrick and Guest 1971; Winslow, Vandegriff et al. 1997). In a molecular setting, it typically blocks infection of most cells by HSV, HIV, Sendai virus, HHV-7, classical swine fever virus, hemorrhagic septicemia virus, and African swine fever virus (Marchetti, Pisani et al. 1995; Fabregas, Garcia et al. 1999; Zhang, Schols et al. 1999; Hulst, van Gennip et al. 2000; Moulard, Lortat-Jacob et al. 2000). For these reasons, it has recently been investigated in a therapeutic light for the prevention and control of HIV disease (Hiebert, Wice et al. 1999; Piret, Lamontagne et al. 2000). On the other hand, target cells resistant to HSV infection often display altered or reduced GAGs on the cell surface. In these cases, DS is able to act as an artificial receptor to mediate infection of cells normally resistant to HSV infection (Dyer, Banfield et al. 1997; Yeung, Bockhold et al. 1999)(Chapter 3). Therefore DS was examined for its capacity to improve tumor regression in a particularly aggressive cancer model by targeting systemically delivered HSV to tumor while minimizing interactions with substances and cells encountered in circulation.

In this study, we show that the addition of DS to the viral inoculum significantly improves the therapeutic index of oncolytic HSV vectors. DS alters the hemodynamics of viral therapy in favor of tumor regression and prolonged survival of tumor-bearing animals in both solitary and metastatic tumor models. Furthermore, we show that DS
increases both the bioavailability of the HSV to tumor and the residence time of active virus \textit{in vivo}. In addition, DS targets oncolytic HSV to tumor with a corresponding decrease in viral accumulation in the liver. The improved delivery of HSV by the addition of DS is evidenced by substantial peripheral degeneration of the tumor. Moreover, lower effective viral doses can be used in combination with DS to achieve significant antitumor efficacy. These results are remarkable and demonstrate that a simple formulation change to current generation HSV vectors increases antitumor efficacy for effective systemic cancer therapy without the complications associated with surgical procedures, increasing viral titers, or modifying viral genomes.
4.2 Results

4.2.1 In vitro and in vivo replication of HSV in CT26 cells

Following systemic administration, HSV is able to reach and infect distant target tumor tissue, in both flank and liver metastatic tumor models (Kooby, Carew et al. 1999; Walker, McGeagh et al. 1999). To characterize the CT26 tumor model with respect to HSV oncolytic therapy, we assessed replication, intravenous (i.v.) delivery, and dosing using NV1020. NV1020 is a replication-competent HSV vector containing only one copy of γ34.5 and a deletion in the terminal repeats such that rearrangement of genome segments is ablated (Meignier, Longnecker et al. 1988; Meignier 1991). This oncolytic vector is currently being evaluated as a candidate for clinical trial.

To determine whether CT26 cells were susceptible and permissive to HSV, we examined in vitro and in vivo replication of HSV in both CT26 cells and established tumors. One step growth curves were plotted to monitor replication of HSV over time in CT26 and Vero cells in culture. To assess replication of HSV in vivo, NV1020 was injected into established CT26 flank tumors, harvested at various time points, and titered. HSV does not replicate efficiently in CT26 tumor cells in vitro even at a high multiplicity of infection (MOI = 10) (Figure 4.1, a). In vivo, the amount of HSV within tumors rapidly declines by day 1 and is undetectable by day 10 post-inoculation (Figure 4.1, b).
Figure 4.1 Replication of NV1020 in vitro and in vivo in CT26 tumor cells.

The top panel, a, details a one step growth curve of NV1020 replication over time in CT26 and Vero cells in culture at high and low MOI. To assess in vivo replication, animals harboring CT26 flank tumors were injected intratumorally with NV1020 (1 x 10^7 PFU). At specific time points, tumors were harvested and titered for active virus. The lower panel, b, describes the in vivo replication of NV1020 in CT26 tumors.
4.2.2 Systemic delivery of HSV to treat CT26 tumors

To evaluate the efficacy of HSV delivered systemically, we established CT26 flank tumors in BALB/c mice and compared systemic delivery with intratumoral injection. NV1020 ($10^7$ PFU) was delivered either by tail vein in a volume of 100 μl, or by direct injection into tumor in a volume of 25 μl. Treatment with virus significantly ($p < 0.02$) reduced mean tumor volume by greater than 50% irrespective of route of administration (Figure 4.2).
Figure 4.2 Efficacy of systemically delivered NV1020 in the CT26 flank tumor model.

CT26 tumors were seeded in BALB/c mice. At a tumor volume of approximately 200 mm$^3$, $10^7$ PFU NV1020 was administered in one dose either intratumorally or systemically (intravenous by tail vein).
4.2.3 Dosing regimen of oncolytic HSV

Multiple intratumoral injections of virus typically show improved efficacy when compared to a single injection of virus (Walker, McGeagh et al. 1999; Delman, Bennett et al. 2000; Lambright, Force et al. 2000). In addition, a number of recent publications indicated that HSV seropositivity should not deleteriously affect the efficacy of HSV oncolytic therapy (Delman, Bennett et al. 2000). Therefore we investigated whether fractionated dosing would also improve efficacy in a systemic model of delivery. CT26 flank tumors were established in BALB/c mice. The first treatment group consisted of mice administered with three doses (2 days apart) of $1 \times 10^7$ PFU of NV1020, while the second group was given a single dose of $3 \times 10^7$ PFU of NV1020. Fractionated dosing of virus significantly increased antitumor efficacy ($p < 0.05$) (Figure 4.3).
Figure 4. 3 Fractionated dosing of NV1020 in the CT26 flank tumor model.

Mice harboring CT26 flank tumors were treated with either a single dose ($3 \times 10^7$ PFU) of NV1020 or three doses ($1 \times 10^7$ PFU) of NV1020 every 2 days.
4.2.4 DS improves HSV oncolytic activity

CT26 flank and metastatic tumor models were established in BALB/c mice to test the antitumor efficacy of systemically delivered NV1020 for the treatment of solid tumors and microscopic disease respectively. The flank model assesses the virus’ ability to limit the growth of an already established tumor. Treatment in the metastatic model is administered 24 hours following the delivery of tumor cells, therefore this model assesses the virus’ ability to inhibit the formation of tumor nodules.

The animals harboring tumors in each of these models were treated with NV1020 in the presence or absence of 100 μg/ml DS at 3 doses of 10^7 PFU every 2 days. Efficacy, as measured by rate of tumor growth or average nodule count respectively, was significantly improved in both tumor models following treatment (p < 0.03, flank model; p < 0.05, metastatic model) (Figure 4. 4). Additionally, survival of tumor-bearing animals was significantly prolonged, with 25% of the animals cured when administered with NV1020 and 100 μg/ml DS (Figure 4. 5). At 9 months following termination of the experiment (efficacy), cured animals still show no tumor. These results demonstrate that DS added to the virus inoculum enhances efficacy and survival in a CT26 flank and metastatic tumor model.
Figure 4.4 Efficacy of dextran sulfate-coated NV1020 on CT26 tumors.

Both panels detail the effect of combining dextran sulfate with the viral inoculum on the treatment of CT26 tumors. For the flank tumor model, upper panel, efficacy is scored by tumor volume. Treatment is given by tail vein every 2 days for a total of 3 doses of $1 \times 10^7$ PFU. The liver metastatic model involves a splenic injection of CT26 cells, which consequently metastasize to the liver. In this model, lower panel, efficacy is scored by average nodule count in the liver. Treatment is administered via splenic injection at one dose of $1 \times 10^7$ PFU.
Figure 4.5 Survival data for animals treated with NV1020 with or without DS.

This graph details the data from the flank tumor model. Eight animals per treatment group; complete experiment was repeated no less than 8 times.
4.2.5 Lowering the effective dosage of oncolytic HSV with DS

The data so far indicated that DS was able to significantly improve antitumor efficacy in the CT26 tumor model when co-administered with a dose of $10^7$ PFU NV1020. Therefore we wanted to determine whether DS could be applied to lower doses of virus to achieve similar levels of efficacy. In the CT26 liver metastatic tumor model, a dose of $10^5$ PFU of NV1020 with or without 100 µg/ml DS was delivered by splenic injection. Doses of $10^5$ PFU NV1020 alone were ineffective at reducing the number of tumor nodules formed. By contrast, when DS was added to the viral inoculum, significant antitumor efficacy was achieved ($p < 0.05$) and the average nodule count was significantly decreased (Figure 4.6). Thus the addition of DS results in antitumor efficacy by two log lower viral doses shown to be ineffective at reducing tumor nodule formation in the CT26 metastatic model.
10^5 PFU of NV1020 had been shown previously to be ineffective at reducing average nodule count in the liver metastatic tumor model. DS was added to the inoculum of 10^5 PFU NV1020 to determine whether lower doses of virus can be administered for oncolytic activity.
4.3 Discussion

Growing tumor cells feed off newly synthesized and recruited vasculature, which is directly accessible by i.v. delivered viral therapeutics. Thus the same portals that the tumor synthesizes to allow for its continued growth can be exploited for systemic cancer therapy. Recent experiments with HSV delivered by the hepatic artery or portal vein in rodents for the treatment of hepatoma or colon carcinoma metastatic to the liver show a profound reduction in tumor nodule formation (Kooby, Carew et al. 1999). Thus antitumor efficacy with loco-regional delivery can be achieved in animal models of cancer. More recently, HSV has been delivered into the tail vein of mice in an attempt to treat prostate tumors in a xenograft model of disease (Walker, McGeagh et al. 1999). I.V. delivery is particularly attractive since surgery is not required and dosing can be specifically tailored during the course of treatment. Furthermore, i.v. oncolytic therapy could easily be administered on an outpatient basis or as an adjunct to standard protocols of care.

The CT26 solid and metastatic tumor models are typically difficult to treat because HSV replication is limited in this cell type. Furthermore, large quantities of virus available to infect tumor cells are thought to be required to see antitumor efficacy. Therefore this model was appropriate to test a novel formulation change to oncolytic HSV for its ability to substantially improve systemic delivery. In this aggressive model, we are able to achieve efficacy with systemic delivery of HSV. DS may direct more virus to the tumor site or package virus in a form better able to destroy tumor cells. Additionally, it may play a role in modulating the immune response toward tumor eradication.

DS is a highly sulfated GAG analog with several interesting properties. Due to its large molecular weight and highly negative charge, it has been shown to interact effectively with surface glycoproteins of many viruses. In this light, it has been largely characterized as an inhibitor of viral infection as it effectively prevents viral glycoprotein interaction with cell surface GAGs for initial attachment. By contrast, DS has been shown to improve transduction levels of certain cell types that are not normally permissive for viral infection. For instance, HSV infection of sog9 cells and mature
myofibers, both of which are relatively devoid of cell surface glycosaminoglycans, is
stimulated by DS (Dyer, Banfield et al. 1997; Yeung, Bockhold et al. 1999) (Chapter 3).
This may be due to DS acting as a surrogate receptor for attachment.

In light of these features, we investigated whether these properties could be
exploited for systemic cancer therapy. In this study, systemic delivery of HSV resulted in
significant antitumor efficacy in the CT26 models of colorectal cancer tested. Our results
indicate that surprisingly little virus is needed for antitumor efficacy in a systemic model
of delivery. When DS is combined with HSV prior to systemic delivery, tumor
regression is further enhanced. Remarkably, this was comparable to direct intratumoral
injection. A corresponding increase in survival is seen with 25% of tumor-bearing
animals cured. It is also important to note that the concentration of DS administered (100
μg/ml) corresponds to the dose currently used in the clinic.

The improved efficacy of multiple injections may reflect a synergistic effect of
multiple HSV inoculations inducing a tumor-specific immune response. It could also
reflect increased cell killing due to multiple tumor cell infections over a period of time.
This could be a consequence of saturated available sites for infection. In contrast, gene
therapy studies with adenovirus show that the virus induces a strong immune response
(both neutralizing antibodies and cell-mediated) which blocks subsequent gene delivery
with adenovirus vectors (Yang, Li et al. 1995; Gathery-Segard 1998).

We also found that lower effective doses of HSV could be delivered when
complexed with DS. Specifically, doses of HSV that were not efficacious in the liver
metastatic model exhibited significant reduction in tumor nodule formation when DS was
added to the viral inoculum. Since much less virus (two orders of magnitude lower) is
needed when DS is added for effective cancer therapy, cost and time for manufacturing
viral vectors and consequently cost of viral cancer therapy is reduced. DS has already
been used extensively in the clinic, and its addition to existing viral clinical trials should
be examined.

In this manner, a formulation change to include DS with virus results in a
significantly improved therapeutic index for cancer therapy. Furthermore, DS is already
used in the clinic. Increasing viral titers or modifying viral genomes are routine methods
used to increase the therapeutic index of oncolytic viruses. Since adding DS to the viral
inoculum is an effective means of accomplishing this goal, the other two options mentioned are open to further enhance antitumor efficacy. Taken together, HSV is an ideal vector to be modified by similar formulations to increase antitumor efficacy and prolong survival. The specific mode of action of the improved antitumor efficacy seen with systemic oncolytic HSV and DS therapy is likely multifactorial. Further studies will be required to determine how DS is able to mediate its antitumor effect in combination with HSV. It will be interesting to examine DS viral therapy in the context of immunity and target cell gene expression. Regardless of the precise mode of action, DS addition to the viral inoculum significantly improves antitumor efficacy in the CT26 model. Moreover, DS is inexpensive and already approved for use in the clinic. Therefore HSV is amenable to modification by similar compounds to improve the therapeutic index of viral treatment and should be considered for clinical evaluation in the treatment of tumors.
CHAPTER 5: Mechanism of Action of Dextran Sulfate Mediated Oncolytic Viral Therapy

5.1 Introduction

The development of gene therapy technologies is approaching clinical realization for the treatment of neoplastic diseases. Many vector systems have been tested over the years and viral systems have proved to be effective vectors for the delivery of anti-cancer genes and direct oncolytic activity. Modifications and development of vectors as well as increased knowledge of the antitumor mechanisms will play a significant role in the further advancement of such therapies.

A high transfection efficiency of the tumor was thought to be necessary for any therapy to exhibit antitumor activity. However, we have shown that marked inhibition of tumor growth occurs even with a low infection efficiency (Chapter 4). Therefore any mechanism that explains the antitumor effect of systemically delivered viral vectors must account for its transfer into a low percentage of tumor/peritumoral cells.

In recent years, it has become clear that angiogenesis not only is important in physiological processes such as embryonic development, wound healing, and organ and tissue regeneration, but also plays a pivotal role in tumor progression and metastasis (Hanahan and Folkman 1996). The target of anti-angiogenic cancer treatment is the genetically normal endothelial cell. Therefore, the development of resistance to angiostatic therapy is very unlikely and has not been reported so far (Boehm, Folkman et al. 1997). If a tumor exceeds the size of ~1 – 2 mm, recruitment of new blood vessels is needed (angiogenesis) to prevent tumor cell apoptosis. Tumor cells promote angiogenesis by the secretion of angiogenic factors, in particular basic fibroblast growth factor and VEGF (Folkman and D'Amore 1996). Recently, evidence emerged that angiogenesis is tightly regulated by a balance of activating and inhibiting factors (Folkman 1995). Therefore, an upset in this balance should counteract the tumor-induced angiogenesis. Furthermore, many reports have indicated that few endothelial cells need to be disrupted in order to instigate vast oncolysis. Since endothelial cells do not vary
from one tumor type to the other, the clinical relevance of such an anticancer approach should be emphasized.

Previous studies have shown marked tumor destruction when oncolytic HSV was administered by a systemic route. The aim of this study is to provide a rationale and working model for the profound oncolysis seen in our cancer models, despite low amounts of virus at the tumor site and a cancer cell type that does not readily support replication of HSV. By examining the properties of DS and HSV with respect to efficacy, pharmacokinetic profiles and distribution, we propose that systemically delivered oncolytic HSV is able to mediate improved oncolysis primarily by an attack on tumor endothelial cells. DS serves to enhance this function by passive targeting of HSV to tumor portals and by increasing the bioavailability of HSV in circulation. The knowledge gained by investigating how therapies work not only builds upon our understanding of tumor biology and viral therapies, but it can be consequently exploited to achieve even higher levels of efficacy on the long road to cures.
5.2 Results

5.2.1 Alteration of the hemodynamic properties of viral therapy

DS has been investigated for its capacity to neutralize virus by coating the virus through ionic interactions with surface glycoproteins (Batinic and Robey 1992; Dyer, Banfield et al. 1997). This has been effective in inhibiting infection of cells normally susceptible and stimulating those typically refractory to viral infection (Batinic and Robey 1992; Dyer, Banfield et al. 1997; Yeung, Bockhold et al. 1999). Therefore we wanted to determine whether DS, complexed with virus, would direct HSV to tumor and protect the virus in circulation from inactivation and unwanted interactions with other substances.

To determine whether DS was able to direct HSV to tumor, \(^{35}\text{S}\)-methionine-labeled NV1020 in the presence or absence of 100 \(\mu\text{g/ml}\) DS was injected by tail vein into animals previously established with CT26 flank tumors. At 2 h post injection, the amount of radioactive HSV in blood, tumor, and major organs was determined. Interestingly, only tumor and liver tissue displayed a significant alteration in radioactivity with DS. There was a 3-fold increase in radioactivity in the tumor with a corresponding 3-fold decrease in radioactivity in the liver (Figure 5.1). These data suggest that when DS is added to the viral inoculum, more virus reaches tumor selectively over time while less virus accumulates in the liver.

To determine whether DS increased the residence time of active HSV in circulation by providing a protective coat, we conducted a pharmacokinetic study. NV1020 with or without 100 \(\mu\text{g/ml}\) DS was injected by tail vein followed with blood sampling at various time points. Circulation time of active virus was increased by 60 min when NV1020 was pre-coated with DS (Figure 5.2). Thus the bioavailability of viral therapeutic was increased by DS for oncolytic activity.
Figure 5.1 Targeting of NV1020 to tumor.

Organ-specific targeting was examined following intravenous delivery (by tail vein) of radioactive-labeled NV1020 with or without DS. Two hours following injection of virus, several organs including tumor were harvested and levels of radioactivity determined by scintillation. Radioactivity was normalized per gram of tissue, and calculated as % ID/g tissue. ID = injected dose.
Figure 5. 2 Pharmacokinetic Analysis of NV1020 with or without DS.

NV1020 with or without DS was injected by tail vein into BALB/c mice. At several time points (as indicated), blood was sampled and assayed for infectious virus. Y-axis is a measure of the titre by calculating the reciprocal dilution required to see no host cell lysis (vero cells).
5.2.2 Properties of HSV involved in DS-mediated oncolytic activity

To determine whether the observed antitumor efficacy was limited to NV1020 or extended to other HSV vectors of differing strains, a number of HSV vectors were tested in the CT26 flank tumor model. Mice harboring CT26 flank tumors were treated with NV1020, G207, or CgalΔ3 (Table 5.1). NV1020 is a replication-competent HSV vector with only one copy of the γ34.5 neurovirulence gene and deletions in the terminal repeats. G207 is a replication-competent HSV vector with both copies of the γ34.5 neurovirulence gene deleted, and an insertion of \( lacZ \) in-frame with ribonucleotide reductase (ICP6). CgalΔ3 is a replication-defective ICP4' HSV vector with an insertion of \( lacZ \) under the control of the HCMV promoter at IE3. All three HSV constructs had comparable oncolytic activity (p < 0.05) (Figure 5.3). Furthermore, the efficacy of all vectors was significantly improved by the addition of DS to the viral inoculum (p < 0.05). Therefore the strain and vector differences did not appear to influence the efficacy of HSV oncolytic activity in this cancer model.

As illustrated in previous work (Chapter 4), profound antitumor activity resulted from i.v. delivery of NV1020. To examine whether viral replication was required to produce the antitumor effects, CT26 flank tumors were established in BALB/c mice and treated with either replication-competent (NV1020) or replication-defective (CgalΔ3) virus. Tumor-bearing mice treated with either of these HSV vectors yielded similar results (p < 0.05) (Figure 5.3). Therefore viral replication was not required for oncolysis by systemically delivered HSV.

In a similar experiment, animals were administered with 4 µM acyclovir (ACV) through their drinking water one week prior and throughout the course of viral treatment. Acyclovir is a nucleoside analog that inhibits replication of HSV. HSV encodes a thymidine kinase that converts ACV to a toxic metabolite (obligate DNA chain terminator). Tumor-bearing mice given ACV alongside viral therapy showed marked reductions in mean tumor volume over time, despite limited viral replication (Figure 5.4). Interestingly, ACVHSV/DS treatment resulted in a greater delay of tumor growth when compared to HSV/DS. However, no cured animals were observed with either replication-defective vectors or when ACV was administered. Taken together, these
results strongly suggest that viral replication is not necessary for the antitumor effect of systemically delivered HSV.
Table 5.1 Description of HSV vectors used in CT26 animal models.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV1020</td>
<td>F</td>
<td>Replication-competent, deletion in terminal repeats</td>
</tr>
<tr>
<td>G207</td>
<td>F</td>
<td>Replication-competent, $\gamma 34.5^*$, insertion of lacZ in-frame with ribonucleotide reductase (ICP6)</td>
</tr>
<tr>
<td>CgalΔ3</td>
<td>17</td>
<td>Replication-defective, insertion of lacZ in-frame with ICP4</td>
</tr>
</tbody>
</table>
Antitumor efficacy of different viral vectors in the CT26 colorectal cancer model.

Animals were seeded with CT26 flank tumors and treated by i.v. with different HSV vectors. Refer to Table 5.1 for detailed descriptions of individual vectors. For clarity, error bars were not included. Measurements did not exceed +/- 10% (control) and 5% (treatment). However, p-values were calculated (see text for details).
Figure 5.4 Antitumor efficacy of NV1020 in the presence of ACV.

A group of mice harboring CT26 tumors were fed 4 μM ACV in their drinking water to prevent viral replication in host cells. One week into ACV administration, animals were treated with NV1020 with or without DS. Control groups were given ACV.
5.2.3 DS inhibits infection of CT26 cells

DS is typically an inhibitor of viral infection. In the case of HSV, DS likely inhibits the interaction between cell surface HS and glycoprotein B for initial attachment. This step is necessary for the virus to interact with secondary protein receptors prior to viral entry. However, for cells that are normally refractory to HSV infection, DS has been found to mediate infection by acting as a surrogate receptor. In this manner, DS serves to tether the virus to the cell surface in the absence of HS.

To determine whether DS was specifically targeting systemically delivered HSV to tumor target cells, cultures of CT26 cells were challenged with DS-coated HSV. CT26 cells are moderately susceptible to HSV infection and consequently, DS inhibited infection of CT26 cells by HSV (G207) (Figure 5.5).
Figure 5.5 Infection of CT26 by DS-coated HSV.

In the left panel, L, CT26, and sog9 cells were infected with β-galactosidase-expressing HSV vectors (G207) (MOI = 0.1). L cells are normally susceptible HSV infection. On the other hand, sog9 cells do not display cell surface GAGs, thereby limiting HSV infection in these cells. In the right panel, L, CT26, and sog9 cells were challenged with DS-coated G207. Cells were examined 16 h following infection. Infection is scored by number of blue cells in the culture dish.
5.2.4 Properties of DS involved in DS-mediated oncolytic activity

The biological properties of DS are closely tied to its concentration and composition: molecular weight (MW) and degree of sulfation (Derrick and Guest 1971; Flexner, Braditch-Crovo et al. 1991; Dyer, Banfield et al. 1997; Witvrouw and De Clercq 1997). For instance, low doses of DS of high MW and sulfation are required to stimulate infection of cells typically resistant to HSV (Dyer, Banfield et al. 1997). Furthermore, different MWs will confer different hemodynamic properties of the circulatory system (Derrick and Guest 1971; Baron and Treib 1998). Thus by investigating which properties of DS play an important role in improving the therapeutic index of oncolytic HSV, we can delineate hypotheses for its specific mode of action in our cancer models.

To determine whether antitumor efficacy of HSV/DS correlated with the concentration of DS administered, CT26 flank tumors were set up in BALB/c mice. Groups were treated with NV1020 and differing concentrations of DS. Antitumor efficacy increases with increasing concentrations of DS (Figure 5.6). The highest dose tested was 500 µg/ml DS, which corresponded to the most effective concentration of DS for antitumor efficacy. The p-values were significant only when 10 µg/ml DS was compared with 500 µg/ml. However, repeated experiments consistently recapitulated this trend. Some toxicity, in the form of excessive bleeding, was seen in animals at 500 µg/ml DS.

Next, we examined whether sulfation was a factor in the mechanism of action of DS by comparing the antitumor efficacy of dextran (unsulfated DS) and DS with NV1020 in the CT26 flank tumor model. Both molecules were equally effective at enhancing the antitumor efficacy of NV1020 (p < 0.05) (Figure 5.7).

A pharmacokinetic study was also conducted with NV1020 and DS of varying concentration, MW, and degree of sulfation. As seen in efficacy studies, dextran and DS were both able to increase the circulation time of active virus over time. The 500 µg/ml dose of DS and 100 µg/ml DS increased circulation times to similar degrees. As well, higher MW DS showed increased circulation times when compared to shorter chain lengths (Table 5.2).
Figure 5.6 Concentration of DS and antitumor efficacy in the CT26 tumor model.

Mice harboring CT26 flank tumors were treated with NV1020 and increasing concentrations of DS. The highest dose tested was 500 µg/ml DS (500DS). 10DS, 10 µg/ml DS; 100DS, 100 µg/ml DS. Error bars were excluded for clarity. However, p-values were calculated (see text for details).
Figure 5. The effect of sulfation of DS on antitumor efficacy.

Animals harboring CT26 flank tumors were treated with NV1020 coated with either 100 μg/ml DS or 100 μg/ml dextran.
Table 5.2: Circulation time of active virus following i.v. delivery of HSV with DS of differing properties.

<table>
<thead>
<tr>
<th>Therapeutic tested</th>
<th>Average Circulation time of active virus (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV1020</td>
<td>55</td>
</tr>
<tr>
<td>NV1020 + 100 µg/ml DS</td>
<td>120</td>
</tr>
<tr>
<td>NV1020 + 500 µg/ml DS</td>
<td>120</td>
</tr>
<tr>
<td>NV1020 + 100 µg/ml dextran</td>
<td>120</td>
</tr>
<tr>
<td>NV1020 + 100 µg/ml low MW DS</td>
<td>90</td>
</tr>
</tbody>
</table>

Values did not exceed an error margin of 10%.
5.2.5 The role of host immune responses in viral oncolysis

The striking antitumor effects observed as a result of fractionated intravascular administration of NV1020 could theoretically be a consequence of viral replication and oncolysis or, alternatively, could be a result of antitumor immune responses. Previous results (refer to Section 5.2.2) indicated that viral replication was not required to produce the antitumor effects observed in vivo.

To determine whether an intact immune system was necessary for the antineoplastic effects of NV1020 oncolysis, subcutaneous CT26 flank tumors were established in immune-incompetent, athymic BALB/c (nu/nu) mice. Established tumors were treated with fractionated i.v. doses of NV1020 with or without 100 µg/ml DS (Figure 5.8). Treatment with NV1020 significantly reduced mean tumor volume by greater than 50% at 9 days following the first dose of oncolytic virus (p < 0.04). With the addition of 100 µg/ml DS to the viral inoculum, significant antitumor effects are also seen (p < 0.03). These results suggest that the intact immune system in BALB/c mice neither enhances nor attenuates the oncolytic effects of NV1020, and that DS can also be used in these animals to improve the therapeutic index of NV1020.
Figure 5. Host immune responses against tumor.

CT26 cells were inoculated into the flanks of athymic BALB/c (nu/nu) mice. Tumors were then treated by i.v. delivery of NV1020 with or without DS. Flank tumor size was measured every 3 – 4 days.
5.2.6 Cured animals display immunity against CT26 tumors

Only the group of animals treated with DS-coated NV1020 displayed a 25% survival of cured animals out to 9 months. To determine whether cured animals had acquired a long-term immunity to CT26 tumors, they were rechallenged with CT26 cells (1 x 10^5) by subcutaneous implantation. The number of CT26 cells (1 x 10^5) used in the initial seeding of tumors was consistent for this experiment. None of these animals displayed tumor up to 6 months post-inoculation (Table 5. 3). On the other hand, animals vaccinated with UV-inactivated CT26 cells developed tumor 7 – 10 days post-inoculation. These data indicated that the antitumor immunity resulting from cured animals is mediated by NV1020.
<table>
<thead>
<tr>
<th>Condition of Animals</th>
<th>Animals with Tumor Following CT26 Rechallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice cured of CT26 flank tumors by NV1020 – DS therapy</td>
<td>0/12</td>
</tr>
<tr>
<td>Mice vaccinated with UV-inactivated CT26 cells</td>
<td>9/10</td>
</tr>
</tbody>
</table>

Table 5.3 Immunity following tumor eradication by DS-coated NV1020.
5.2.7 Histological analysis of tumors treated with DS-HSV

Based on the improved tumor-specific delivery and increased bioavailability of HSV complexed with DS, we investigated whether this would correspond to greater peripheral damage of established tumors. Upon histological analysis of tumors treated with systemic delivery of NV1020 and 100 ug/ml DS, greater peripheral degeneration of tumor was seen when compared to NV1020 administered alone (Figure 5. 9). Interestingly, apoptotic cells were distributed over the periphery of the tumor, rather than at a localized area.
Figure 5.9 Histology of tumors treated with i.v. dosing of NV1020.

Animals harboring CT26 flank tumors were treated with $10^7$ PFU NV1020 with or without DS by i.v. delivery (tail vein). Two days post injection, tumors were removed, frozen in OCT, and sectioned. Samples were stained with hematoxylin and eosin. Lighter shaded regions are typical of cells undergoing apoptosis (see arrows). These sections are representative of histology seen from 3 animals per treatment group.
5.2.8 Oncolytic HSV localizes to tumor vasculature

The results thus far suggested that the widespread oncolysis observed by i.v. delivery of NV1020 with DS may principally involve destruction of tumor vasculature. Systemic delivery of NV1020 with DS is as effective as intratumoral injection of NV1020. In addition, viral replication is not necessary for the oncolytic activity of NV1020. Furthermore, extensive peripheral degeneration is seen with systemic delivery of HSV.

Angiogenesis, the sprouting of new blood vessels from the pre-existing vessel bed, is an absolute requirement for the growth of tumors beyond $1 - 2 \text{ mm}^3$ in volume. About 100 tumor cells rely on every endothelial cell for the delivery of nutrients, therefore not all endothelial cells need to be damaged to achieve profound tumor responses. Effecting only a few endothelial cells can shut down the blood supply to the entire tumor. It is possible that the low amounts of virus are able to result in tumor kill despite replication because they are primarily effecting tumor endothelial cells.

To determine whether tumor endothelial cells were in fact being infected by NV1020, virus was delivered intravascularly to mice harboring large liver tumor nodules. In order to monitor the cells infected by NV1020, the $gfp$ gene was inserted into one of the terminal repeats of the NV1020 backbone (termed NV1066). This virus was tested in both CT26 animal models and was found to retain antitumor efficacy. At 48 hours post-delivery, livers were harvested and frozen in OCT. Sections were taken and CD31 immunohistochemistry was performed. NV1066 localizes to tumor endothelial cells (Figure 5. 10). Furthermore, infection of tumor tissue is localized to the vicinity of tumor vasculature. As well, all observed infected areas co-localized with a tumor vessel. Consistent with our results, the vector does not appear to spread extensively.
Figure 5. 10 CD31 immunofluorescence of infected tumor endothelial cells.

CT26 liver tumor nodules were established in BALB/c mice by splenic injection of 1 x 10^5 CT26 cells. At approximately 2 weeks post inoculation, NV1066 was delivered intravascularly. At 48 h post delivery, animals were sacrificed and livers removed for immunohistological analysis. Infected tumor cells are indicated by GFP expression (green areas). Endothelial cells were identified by mouse anti-rat CD31 antibodies and visualized by goat anti-mouse Cy-5 antibodies (red areas). Yellow areas show co-localization of virus and endothelial cells, indicating successful infection by HSV.
5.3 Discussion

Several biological properties of HSV render it an ideal vector for cancer therapy (Breakefield, Kramm et al. 1995; Jacobs, Breakefield et al. 1999). First, tumor cells infected with HSV are destroyed during viral replication; transgene expression is not required for antitumor activity (Mineta, Rabkin et al. 1994; Carroll, Chiocca et al. 1996). Second, these oncolytic effects are present even at extremely low MOI compared to adenovirus, vaccinia virus, and reovirus (Boviatsis, Scharf et al. 1994; Carroll, Chiocca et al. 1996). Third, HSV's intrinsic thymidine kinase is expressed during viral replication, thereby permitting enhancement of the antitumor effect by treatment with prodrugs such as ACV (Jacobs, Breakefield et al. 1999). Fourth, HSV-1 vectors can carry a large payload, thereby allowing delivery of multiple therapeutic genes to increase oncolytic activity (Roizman and Sears 1996). Lastly, the virus is a common pathogen in humans, yet very rarely causes serious medical illness (Roizman and Sears 1996).

In this study (Chapter 4), we have demonstrated the therapeutic efficacy of systemically delivered NV1020 complexed with DS for the treatment of colorectal metastases to the liver. Our studies have indicated that very little virus is required for oncolysis and eradication of tumors. In order to better understand the mechanism of action behind this efficacy in our animal models of cancer, viral properties, DS properties, immunity, and target cells were analyzed to formulate a working model. Consequently, this model can be exploited to improve upon existing experimental treatment regimens to further increase the therapeutic potential and safety of oncolytic HSV vectors.

Interestingly, DS appears to alter the hemodynamic properties of viral therapy and thereby addresses a number of concerns with respect to i.v. delivery. Preliminary studies have yielded a number of postulations to suggest that DS may serve to direct oncolytic virus to tumor, although DS is typically an inhibitor of viral infection. Our data indicate that systemic delivery of HSV with DS results in 3-fold higher accumulation of virus in the tumor and a 3-fold decrease of virus in the liver. This finding is particularly significant as potential liver toxicity is decreased and antitumor efficacy is increased by the addition of DS.
Moreover, coating HSV with DS prior to delivery increases the bioavailability of HSV to tumor over time. We show that the addition of DS to the inoculum results in an increase in circulating active virus by 60 min. Based on the cardiac output of a mouse, this translates to approximately a 200-fold increase in number of passes that the virus makes at the vascular entry site of tumor. Thus DS could also be acting as a protective molecule against cells and other substances encountered in circulation that rapidly inactivate viral particles. DS is a negatively-charged molecule that interacts with the positively-charged glycoproteins that stud the viral surface. Through this ionic interaction, DS coats the virus, thereby preserving its activity in circulation while likely preventing adherence of virus to vessel walls or cells. As tumor vasculature is leaky and has different properties compared to the vasculature of other tissues, the net effect may result in a greater accumulation of virus at the port of entry to the tumor (passive targeting). Together, these measures alter the hemodynamic properties of HSV in circulation such that the innate ability of oncolytic HSV to infect tumor is enhanced.

Upon investigation of the viral properties responsible for oncolytic activity, we encountered an unexpected result. Although differing strains and constructs of HSV vectors were amenable to antitumor therapy with DS, viral replication was not necessary for HSV-mediated oncolysis. This was shown by using replication-defective viruses and also by the concomitant administration of ACV during treatment with replication-competent viruses. This data corresponds to earlier experiments that showed limited replication of HSV in CT26 cells. It is well established that oncolysis by direct injection into a tumor requires high doses of replication-competent viruses. As a result, this data suggested that tumor cells were not likely the primary target cells responsible for the observed profound tumor destruction. Rather, we hypothesized that the primary targets were likely the tumor support cells such as tumor endothelial cells. Due to an amplification effect, it is well known that only a handful of tumor endothelial cells need to be destroyed in order to result in massive tumor kill.

Yet another puzzling observation was that ACV appeared to potentiate oncolysis. Entry of HSV into tumor cells results in viral gene expression followed by cellular lysis. HSV encodes a TK that converts acyclovir to a toxic metabolite. This metabolite may be transferred between cells and lead to the death of neighboring uninfected cells, termed
bystanders. A number of studies have demonstrated a correlation between the degree of gap junction-mediated intercellular communication in tumor cells and the extent of ACV-mediated bystander killing (Fick, Barker et al. 1995; Elshami, Saavedra et al. 1996; Mesnil, Piccoli et al. 1996). Once ACV molecules are phosphorylated by viral TK, they cannot diffuse through the plasma membrane. However, phosphorylated ACV can be transferred to adjacent cells via gap junctions resulting in the death of uninfected cells. Gap junction-mediated intercellular communication is impaired in many tumors (Yamasaki, Mesnil et al. 1995), and thereby potentially poses a major limitation to ACV-mediated bystander killing. In these cases, investigators have transfected tumors with gap junction proteins and demonstrated enhanced bystander effect in the transfectants (Elshami, Saavedra et al. 1996; Mesnil, Piccoli et al. 1996).

This study has demonstrated profound oncolysis following systemic administration of NV1020 with DS, which was significantly increased concomitant with ACV administration. However, colorectal cancer cell lines typically have a lack of bystander effect due to impaired gap junction-mediated intercellular communication (Yamasaki, Mesnil et al. 1995). In HSV-infected cells, viral TK phosphorylates ACV to the monophosphate form. Cellular enzymes convert the monophosphate to ACV triphosphate, which is the actual inhibitor of DNA synthesis (Wood 1996). The lack of bystander effect observed in colorectal cancer cells could be explained by a deficiency or dysfunction of cellular enzymes necessary for the formation of the triphosphate. Therefore the cell would be resistant to the prodrug and no bystander killing could take place. Thus the extensive damage seen in our model could be attributed to efficient gap junction-mediated intercellular communication between target endothelial cells. This data is consistent with our hypothesis that the primary target cells are likely the tumor endothelial cells.

DS is a versatile molecule with a wide range of biological properties (Chapter 1.15). These properties vary as a function of its sulfation, MW, and concentration. For instance, Dyer and colleagues found that 10 µg/ml of DS (500,000 MW) was optimal for stimulating the infection of cells normally resistant to HSV-1 infection (Dyer, Banfield et al. 1997). This was also confirmed in the skeletal muscle study, which showed that DS could also be used to stimulate infection of mature skeletal muscle typically refractory to
HSV-1 infection (Yeung, Bockhold et al. 1999) (Chapter 3). These resistant cells were deficient in HS synthesis and therefore it was proposed that DS was required to interact with a scavenger receptor and viral glycoproteins for initial attachment. This would serve to stabilize the virus for stable attachment and consequently productive infection.

On the other hand, DS has been used routinely for years in the management of blood volume and perfusion of tissues (Chapter 1.15.2). As opposed to its uses in molecular virology, its effects on the hemodynamic properties in blood circulation increases with increasing dosages. Moreover, sulfation is not required to modulate circulation. Since, the properties of DS are tied to its function in different systems, we examined these properties to determine how DS was able to bring about high levels of antitumor efficacy. Previous experiments had indicated that CT26 cells were not stimulated by DS. Rather, DS inhibited infection of CT26 cells by HSV-1. In addition, higher concentrations of DS showed improved antitumor efficacy in the CT26 flank tumor model. Furthermore, either dextran or DS administered with HSV-1 resulted in massive oncolysis to similar degrees. Thus sulfation was not necessary to bring about antitumor efficacy. Tumor vasculature has been described as embryonic in structure and by consequence leaky. Therefore, the expansion in blood volume by DS, along with the ability of DS to protect HSV-1 in circulation, may result in selective uptake of HSV-1 by tumor support cells. Taken together, these results suggest that DS is acting non-specifically to alter the hemodynamic state in favor of selective extravasation at the tumor site.

In accordance with our hypothesis, greater peripheral degeneration is seen in tumors treated systemically by HSV with DS as compared to HSV without DS. This could be attributed to an increase in local blood pressure since DS is commonly used as a volume expander. Since tumor vasculature is characteristically leaky (Hashizume, Baluk et al. 2000), an increase in extravasation of oncolytic HSV to tumor tissue may result. This finding also corresponds well with the above-mentioned postulations (Figure 5.1, Figure 5.2) which both demonstrate a local accumulation of active oncolytic virus at the tumor site. To test this directly, CD31 immunofluorescence was conducted on tissue sections of liver containing large tumor nodules. Co-localization of virus to tumor
vasculature was seen, lending strong support to tumor endothelial cells as primary targets for systemically delivered oncolytic HSV.

The benefits that arise from the study of the mechanism of action for therapies are numerous. Not only can we learn more about the biology of vector and animal systems, but we can also exploit that knowledge to formulate improved treatments for disease. The data from this study point to a multi-mode of action for DS in the treatment of both flank and metastatic liver cancer. Firstly, DS is able to increase the bioavailability of active virus in circulation over time. In this manner, more virus can be displayed at the site of tumor for oncolysis. This likely occurs through a shielding or protective mechanism to prevent viral inactivation by interaction with molecules encountered in circulation. Furthermore, its properties as a volume expander enable the selective accumulation of virus at tumor sites because tumor vasculature is characteristically leaky. A number of publications have demonstrated the accumulation of macromolecules at tumor portals for these reasons (Jain 1987; Roberts and Palade 1997; Hashizume, Baluk et al. 2000). The net result is the presence of more active virus over time at the tumor site.

Our proposed model for HSV action at the site of the tumor involves primarily infection of tumor endothelial cells. This is not to say that tumor cells are not infected. Some tumor cells are indeed infected; however, HSV does not replicate very well in these cells and therefore infection is limited to the small and localized areas. It is possible that infected tumor cells are induced to secrete anti-angiogenic substances. However, direct injection of high titers of oncolytic HSV do not show the marked peripheral tumor destruction seen with systemic delivery of much lower doses of HSV. Interestingly, HSV-infected areas within the tumor are limited to the periphery and appear to correspond with tumor vessels. Furthermore, systemically delivered HSV is able to directly infect tumor endothelial cells. Endothelial cells display 3-O-sulfated HS, a known cell surface receptor for HSV-1 (Campadelli-Fiume, Cocchi et al. 2000). In addition, we as well as other groups have shown successful infection of endothelial cells with HSV (Vercellotti 1998). As mentioned earlier, only a few endothelial cells need to be destroyed in order to start a cascade of oncolysis (Folkman 1996; Folkman 1997; Folkman 1997). It is likely in this manner that low amounts of virus can result in massive
oncolysis. Although the most prominent effect may be an attack on tumor endothelial cells, the resulting oncolysis seen with systemically delivered HSV likely reflects a combined effect of direct (infection) and indirect tumor cell killing (endothelial cell killing and/or secretion of antiangiogenic substances from tumor cells).

It is well established that prevalence of pre-existing antibodies to HSV-1 does not preclude HSV-1 therapy (Delman, Bennett et al. 2000; Yoon, Nakamura et al. 2000). Furthermore, we have shown that immune-compromised animals can respond to this therapy. Cured animals display lifelong antitumor immunity and consequently do not develop tumor following rechallenge by CT26 cells. Therapies that selectively target vascular endothelial cells are less likely to induce resistance than those that target the tumors themselves. Together with the success of repeated HSV dosing, this would permit longterm maintenance therapy. Intratumoral administration of HSV vectors is currently undergoing various clinical trials for malignant glioma (Phase III). However, instances of metastatic and microscopic disease have demanded the development of systemic modes of delivery that are safe and effective. In the past couple of years, a few groups have shown some efficacy with systemically delivered viral vectors. We have been able to further improve on that through a simple formulation change involving the addition of DS to the viral inoculum. As DS is currently approved for use in the clinic and inexpensive to prepare, its addition to current clinical trial protocols should be considered.
CHAPTER 6: Methods to Improve Delivery of Oncolytic HSV to Its Target – Tumor Vasculature

6.1 Introduction

Angiogenesis, the process of new blood vessel formation from existing vessels, is a fundamental process in cancer development (Folkman 1997). Angiogenesis is a complex process that includes the activation, proliferation, and migration of endothelial cells. In tumorigenesis, this process also involves disruption of the vascular basement membrane, formation of vascular tubes and networks, and linkage to pre-existing vascular networks. In recent years, it has become clear that angiogenesis not only is important in physiological processes such as embryonic development, wound healing, and organ and tissue regeneration, but also plays a pivotal role in tumor progression and metastasis (Hanahan and Folkman 1996). Recent experimental evidence indicates that tumor-related angiogenesis contributes significantly to the malignant phenotypes. The concept of angiogenesis-dependent tumor growth was introduced and experimentally demonstrated by Folkman and others (Brem, Brem et al. 1976; Holmgren, O'Reilly et al. 1995; Hanahan and Folkman 1996; Folkman 1997; Kerbel 1997; Hanahan 1998).

The target of antiangiogenic cancer treatment is the genetically normal endothelial cell. Therefore, the development of resistance to angiostatic therapy is very unlikely and has not been reported so far (Boehm, Folkman et al. 1997). If a cancer exceeds the size of 1 – 2 mm, recruitment of new blood vessels is needed (angiogenesis) to prevent tumor cell apoptosis. Tumor cells promote angiogenesis by the secretion of angiogenic factors, in particular basic fibroblast growth factor and vascular endothelial growth factor (Folkman and D'Amore 1996). Recently, evidence emerged that angiogenesis is tightly regulated by a balance of activating and inhibiting factors (Folkman 1995). Therefore, continuous overexpression of antiangiogenic factors by gene therapy, for instance, should counteract the tumor-induced angiogenesis. This would provide a powerful approach to combat solid tumor growth.

Many tumor- and non-tumor-associated antiangiogenic factors have been described. The proteolytic cleavage of larger precursor molecules associated with the
vascular system (proteins of the coagulation cascade and basement membrane proteins) is thought to play an important role in the generation of several of these antiangiogenic proteins and, thus, in the control of angiogenesis. Some of these include thrombospondin (Good, Polverini et al. 1990; Volpert, Lawler et al. 1998), interferon α (Dvorak and Gresser 1989; Singh, Gutman et al. 1995), and platelet factor 4 (Maione, Gray et al. 1990).

Application of anti-angiogenesis strategies for cancer therapy have been extensively studied and reviewed by Zetter (Zetter 1998). Of the known angiogenesis inhibitors, the recently discovered protein endostatin, a 20-kDa (184 aa) C-terminal fragment of collagen XVIII, is the most potent inhibitor of tumor angiogenesis described so far (O'Reilly, Boehm et al. 1997). Endostatin has been shown to inhibit endothelial cell proliferation and migration, to induce G₁ arrest and apoptosis of endothelial cells in vitro, and to have a potent antitumor effect in vivo in several independent studies (Boehm, Folkman et al. 1997; Blezinger, Wang et al. 1999; Chen, Kumar et al. 1999; Dhanabal, Ramchandran et al. 1999; Dhanabal, Ramchandran et al. 1999; Dhanabal, Volk et al. 1999; Yamaguchi, Anand-Apte et al. 1999). Several cycles of prolonged treatment utilizing systemic administration of purified endostatin resulted in complete tumor regression in several murine tumor models with no drug resistance or side effects (Boehm, Folkman et al. 1997). However, this protein-based therapy is likely to require repeated and long-term administration of high-quality endostatin for optimal therapeutic benefit.

To date, common strategies to block angiogenesis have focused on the administration of antiangiogenic proteins. Human endostatin is rapidly cleared from the blood (O'Reilly, Holmgren et al. 1994; O'Reilly, Boehm et al. 1997). A prolonged exposure of purified angiostatin at high doses was indeed required to maintain cytostatic intratumoral concentrations of angiostatin (O'Reilly, Boehm et al. 1997). Therefore, angiostatic therapy will require a prolonged maintenance of therapeutic levels in vivo. A major difficulty in translating these strategies to the clinic is the unavailability of these proteins in large quantities for chronic treatment. Besides being expensive to increase their production, especially for longterm therapy, protein drugs are unstable in vivo and pose difficulties with frequent readministrations and in the formulation of effective.
sustained-release systems (Putney and Burke 1998). By contrast, the delivery of gene cassettes encoding these factors in appropriate expressible forms offers the potential of enduring benefits from a single treatment or a small number of treatments (Tanaka, Manome et al. 1997).

Direct *in vivo* delivery of the corresponding genes with viral vectors constitutes an attractive solution to this problem. Because most cancer gene therapies currently rely on destructive strategies that target the tumor cells (Roth and Cristiano 1997), viral-mediated gene delivery of an angiostatic factor represents a conceptually different, and possibly synergistic, approach to fight cancer.

Previous studies indicated that endothelial cells were permissive to HSV-1 infection when delivered via a systemic route (Chapter 5). Very low quantities of virus were able to delay tumor growth, and in some instances, eradicate tumors. For these reasons, we hypothesized that the primary mode of oncolysis by systemically delivered HSV was an attack on the tumor vascular network. Based on our proposed mechanism of action of systemically delivered oncolytic HSV, we devised methods to improve this treatment by either targeting virus specifically to tumor vasculature or engineering a virus to secrete antiangiogenic proteins. We reasoned that if the tumor endothelial cells were in fact important targets for HSV-1, directing more virus to the tumor vasculature would result in increased antitumor efficacy. Therefore, to further improve the efficiency of infection of endothelial cells by HSV-1, we coated with virus with targeting peptides aimed at the vascular portals to tumor. In a parallel experiment, HSV vectors containing either angiostatin or endostatin were constructed and tested for their ability to enhance antitumor efficacy.

One of the more challenging aspects of this study is to increase the antitumor therapeutic window. Solid tumors require angiogenesis, the proliferation of blood vessels to grow (Folkman 1995). Antiangiogenic targeting of the vascular compartment of tumors may offer considerable advantage over conventional therapy directed against tumor cells. Recent studies demonstrate the enormous potential of this approach to circumvent the most challenging problems of cancer treatment, including drug resistance and normal tissue toxicity. Crucial to tumor angiogenesis is the balance between tumor-associated angiogenic stimulators, such as VEGF (Hlatky, Hahnfeldt et al. 1996; Brown,
Detmar et al. 1997) and endogenous angiogenic inhibitors, such as endostatin (Boehm, Folkman et al. 1997; O'Reilly, Boehm et al. 1997) and angiostatin (O'Reilly, Holmgren et al. 1994). The ability to modulate levels of these factors may significantly advance cancer treatment by providing a way to regress the tumor via its vascular network.
6.2 Results

6.2.1 The construction of tumor-targeting peptides

The endothelium of solid tumors, while a very attractive potential target for anticancer therapy, has not been well characterized primarily because it has been difficulty to isolate and culture. It has long been known that tumor endothelium divides much faster than most other vascular beds, and it is relatively more leaky (Lazo 1986; Hashizume, Baluk et al. 2000). Tumor endothelium is likely to be exposed to tumor growth factors and to a unique environment associated with tumor physiology. Thus it seems reasonable to assume that biochemical differences may exist between endothelium from malignant and nonmalignant tissues.

Furthermore, there are indications that the vascular beds in different parts of the body are morphologically and functionally different (Risau 1995; Hanahan and Folkman 1996). This is particularly true of the lymphoid tissues, where the high endothelium is composed of cells that express unique adhesion molecules for lymphocyte homing (Streeter, Berg et al. 1988; Lasky 1992; Salmi and Jalkanen 1997). Moreover, metastasis into preferred organs by certain tumors may depend on interactions between tumor cells and organ-specific molecules in vascular beds.

Ruoslht and colleagues used phage display peptide libraries to obtain defined peptide sequences interacting with particular organ and tumor blood vessels (Pasqualini and Ruoslahti 1996). In this system, peptides in as many as $10^9$ permutations are expressed on the phage surface by fusion to one of the phage surface proteins and the desired peptides are selected on the basis of binding to the target molecule. Following several rounds of selection, several targeting peptides were isolated. CNGRCVSGCAGRC corresponded to a tumor-targeting peptide, later shown to interact with a specific subset of integrins on the surface of tumor vasculature (Koivunen, Gay et al. 1993).

In order to coat oncolytic HSV with this tumor targeting peptide, we modified the peptide sequence published by Ruoslht to encode a domain that would interact with DS through charge interactions: CNGRCVSGCAGRCKKKK (Figure 6.1). The arginine and lysine residues of glycoproteins B and C of HSV interact with the highly
negatively-charged DS. This allows DS to effectively coat the virus. Thus the targeting construct was modified to include a number of arginine and lysine residues in the N-terminal region, in addition to the tumor-specific targeting sequence in the C-terminal region. In this manner, the positively-charged N-terminal region of the peptide may interact with DS in an ionic fashion (Figure 6.2). Therefore NV1020 was mixed with DS initially, prior to the addition of the tumor-targeting peptide, in order to allow for the intended charge interactions. Specifically, the final concentrations of the three components in this construct were: (i) $10^8$ PFU/ml NV1020; (ii) 100 μg/ml DS; (iii) 50 ug/ml tumor-targeting peptide. The resulting construct likely was consequently stabilized through charge interactions.
Figure 6.1 Amino acid sequence of the tumor-targeting peptide.

Ruoslahti and colleagues published a peptide sequence capable of targeting molecules to the vasculature of tumors. The most effective sequence is shown in black and red. The red “NGR” sequence is the key element to all tumor-targeting peptides isolated. Marked in blue are the sequences that we added. These positively-charged sequences interact with the negative sulfate groups on DS. In this manner, the peptide can effectively coat HSV/DS. C, cysteine; N, asparagine; G, glycine; R, arginine; V, valine; S, serine; A, alanine; K, lysine.
Figure 6.2 Schematic of tumor-targeting virus construct.

HSV envelope glycoproteins B and C (positively-charged) interact with dextran sulfate (negatively-charged). The N-terminus of the targeting peptide (positively-charged) interacts with dextran sulfate (negatively-charged). This construct allows for tumor-specific targeting of HSV to tumor vasculature via a simple formulation change of the viral inoculum.
6.2.2 The use of tumor-targeting peptides to direct oncolytic HSV

To further improve upon HSV/DS therapy, the tumor-targeting HSV construct was tested in the solid and metastatic colorectal models of cancer. Based on our proposed model that systemically delivered HSV was effecting tumor endothelial cells, we hypothesized that directing virus specifically to the tumorvasculature should allow for increased antitumor efficacy in the colorectal models of cancer. Although the interaction among virus, DS, and peptide was not measured directly, improved oncolysis with this construct and the loss of this efficacy with a lung-targeting peptide suggest that this construct is stable.

In the first model, CT26 flank tumors were established in BALB/c mice. Once tumors reached a volume of approximately 100 mm$^3$, they were treated by a tail vein injection of the HSV tumor-targeting construct every 2 days for a total of 3 treatments (3 @ 1 x 10$^7$ PFU). The tumor-targeting peptides further improved upon HSV/DS treatment of these tumors, allowing for a significant reduction in mean tumor volume over time (p < 0.01 vs. control; p < 0.05 vs. NV1020/DS) (Figure 6.3). In the CT26 metastatic tumor model, mice were seeded with CT26 liver micronodules via splenic injection. A single administration of the HSV tumor-targeting construct (10$^7$ PFU) was given via splenic injection 24 h later. Although marked improvements in antitumor efficacy were observed with the flank tumor model, the tumor-targeting construct did not significantly reduce the development of liver nodules as compared to HSV/DS (Figure 6.4).
Figure 6.3 Antitumor efficacy of the tumor-targeting HSV construct in a solid tumor model.

Mice harboring CT26 flank tumors were treated with the HSV tumor-targeting construct, 3 doses, each 2 days apart.
Figure 6.4 Antitumor efficacy of the HSV tumor-targeting construct in a metastatic tumor model.

BALB/c mice were injected with CT26 cells via splenic injection. 24 h following this injection, they were treated with a single splenic injection of the HSV tumor-targeting construct.
6.2.3 The use of anti-angiogenic viruses to improve oncolytic activity

In another attempt to improve upon HSV/DS oncolytic therapy, HSV vectors were engineered to contain either angiostatin or endostatin genes. The backbone of this virus is G207, which has been shown previously to be as effective as NV1020 in CT26 models of cancer (Chapter 5). The angiostatin or endostatin genes were inserted into ICP6 of G207, instead of the \( \text{lacZ} \) gene. Therefore the resulting vector has both copies of the neurovirulence gene (\( \gamma 34.5 \)) deleted, and a disruption of ribonucleotide reductase (ICP6) gene by either the angiostatin or endostatin gene. DS had already been shown to significantly improve systemic delivery of HSV vectors, thus it was included in the inoculum for all experiments (Chapter 4). Based on our proposed model, administration of HSV vectors encoding anti-angiogenesis factors would further improve HSV/DS therapy.

Mice harboring CT26 flank tumors were treated with a single dose of angiostatin or endostatin encoding viruses \( (10^7 \text{ PFU}) \) by tail vein. Fractionated delivery could not be carried out at this dose because the animals quickly exhibited negative clinical signs (ruffling of fur and lack of activity). The group treated with angiostatin viruses was sacrificed 24 h later. An autopsy revealed liver and kidneys that were pale pink in color, a stark contrast to their typical dark red color characteristic of normal tissue. The group treated with endostatin viruses did not exhibit severe negative clinical signs (slight ruffling of fur), therefore they were monitored closely for the duration of the experiment. A single dose of endostatin HSV \( (10^7 \text{ PFU}) \) resulted in significant antitumor efficacy \( (p < 0.01) \) (Figure 6.5).

Based on these results, a log lower of the anti-angiogenesis viruses were administered by fractionated delivery \( (10^6 \text{ PFU every 2 days for a total of 3 treatments}) \). No negative clinical signs were observed at this dosage. Remarkably, a number of animals were cured at this low dose of virus: 60% of the mice administered with angiostatin HSV were cured and 80% of the mice administered with endostatin HSV were cured (Figure 6.6). Both of these groups exhibited significant antitumor efficacy \( (p < 0.005) \) (Figure 6.5). These results show that repeated cycles of anti-angiogenesis therapy induce tumor dormancy that persists indefinitely after therapy.
Figure 6.5 Antitumor efficacy of anti-angiogenesis viruses in the CT26 flank tumor model.

CT26 flank tumors were established in BALB/c mice. Once the tumors reached volumes of approximately 150–200 mm³, animals were treated with anti-angiogenesis viruses by tail vein. The dosing regimen was every 2 days, for a total of 3 doses, except in the case of $10^7$ endostatin/DS (only one dose was administered).
Figure 6.6  Survival of animals treated with anti-angiogenesis viruses.
Animals cured from these experiments have not shown signs of recurrence out over 40 days.
6.3 Discussion

Tumors are composed of large numbers of cells that have escaped from growth control. Re-establishing growth control or cell killing will significantly influence tumor growth only if the therapeutic agent reaches most or all transformed cells. This is a major limitation for many tumor therapies, especially when solid tumors are considered. Gene therapy makes no exception to this rule. Penetration throughout the tumor is difficult to achieve with viral treatments and viral transduction efficiency is an additional limitation. Therefore gene therapy strategies that provoke bystander effects (i.e. prodrug activating genes) appear more promising than approaches where the antitumoral effect is limited to the cells actually transduced.

Angiogenesis is required for tumors to grow beyond a few millimeters in size (Folkman and D'Amore 1996). Advantages of anti-angiogenic therapy include ease of access of drugs to the endothelial cell compartment, and targeting of a genetically stable cell population (endothelial versus tumor cells) thereby lessening the chance of drug resistance. Furthermore, "amplification" is achieved, because one endothelial cell supports the growth of 100 tumor cells (Bicknell 1994; Burrows and Thorpe 1994; O'Reilly, Holmgren et al. 1994; Baillie, Winslet et al. 1995; Fan, Jaggar et al. 1995).

Previous work (Chapter 5) detailed a mechanism of action for neoplastic HSV, whereby systemically delivered virus destroyed tumor endothelial cells for marked oncolysis. The importance of this proposed model lies not only in an increased understanding of tumor biology and mechanisms for oncolytic viruses, but also in our ability to exploit this knowledge in order to improve the therapeutic index of our treatments. In this light, we modified HSV in several ways to allow for an enhanced attack on tumor endothelial cells. Previous data suggested that it was primarily an attack on the tumor vasculature that led to the remarkable levels of antitumor efficacy and consequently cures. Therefore by either selectively directing oncolytic HSV to the tumor vasculature or increasing endothelial apoptosis via the delivery of antiangiogenic agents, we were able to significantly improve upon the efficacy of oncolytic HSV vectors.

Following the isolation of tumor-targeting peptides, Ruoslahti and colleagues were able to successfully couple these peptides to cancer chemotherapeutics and
demonstrate significant increases in survival of treated animals (Arap, Pasqualini et al. 1998). We therefore modified this peptide to encode a sequence that would bind DS. This peptide may have been able to direct HSV/DS to the tumor vasculature, as supported by a striking increase in antitumor efficacy in the CT26 flank tumor model. In addition to increasing endothelial cell infection by delivering more virus to tumor vasculature, improved oncolysis may also reflect an increase in direct tumor cell infection. Although this may not be as prominent as an attack on the endothelial cells, this could further contribute synergistically to oncolysis.

However, this construct did not improve upon HSV/DS therapy in the CT26 liver metastatic model. As opposed to the flank model, the liver metastatic model involves microscopic disease with little, if any, evidence of developed vasculature. At this stage, a splenic injection of oncolytic HSV is administered to test the prophylactic ability of the virus to prevent the development of tumor nodules, which are then able to effectively recruit their own vasculature from the hepatic artery. Since treatment is administered only 24 h following a splenic injection of tumor cells, we did not expect the targeting construct to improve antitumor efficacy in this model. The absence of tumor vasculature at this stage of disease in this model did not provide a target for our construct, thereby precluding improved delivery of this construct.

Antiangiogenic tumor therapies have recently attracted intense interest for their broad-spectrum action, low toxicity, and, in the case of direct endothelial targeting, an absence of drug resistance. To promote tumor regression and to maintain dormancy, antiangiogenic agents need to be chronically administered. Therefore a potential practical problem to the clinical implementation of dormancy therapy may be the requirement for longterm, systemic drug delivery, a particularly vexing problem for a protein drug like angiostatin or endostatin. Antiangiogenic gene therapy holds promise for circumventing the problems associated with chronic delivery of antiangiogenic proteins (Stratmann, Machein et al. 1997; Tanaka, Manome et al. 1997; Kong, Hecht et al. 1998; Lin, Buxton et al. 1998). Viral vector-mediated targeted anti-angiogenesis may offer a solution to this problem for the treatment of localized areas of pathological angiogenesis such as seen in diabetic retinopathy, rheumatoid arthritis, and cancer. In this manner, viral gene therapy offers a potential way to achieve sustained therapeutic
release of potent antiangiogenic substances if stable and adequate transgene expression can be achieved and maintained for the full duration required for dormancy therapy. Furthermore, angiostatic-based therapies should apply to all solid tumors since endothelial cells do not vary from one tumor type to the other, further emphasizing the clinical relevance of such an anticancer tactic.

We took this approach to enhance endothelial apoptosis in the CT26 flank tumor model. Remarkably, both angiostatin and endostatin encoding viruses resulted in improved antitumor efficacy and survival. Although expression of antiangiogenesis proteins was not confirmed directly in this experiment, prior art on this virus, toxicity exhibited by moderate doses of virus, and the antitumor efficacy of these viruses all suggest that the antiangiogenesis proteins are being expressed. A high dose of angiostatin virus ($10^7$ PFU) was shown to be toxic. Since the livers and kidneys were found to lack blood circulation (pale in color), toxicity was likely due to high levels of angiostatin being produced. The spleen appeared normal, thereby indicating that toxicity was not likely due to a severe infection (bacteria, impure preparations of virus). As a result, we administered a log lower virus ($10^6$ PFU), which resulted in a profound reduction in mean tumor volume and a corresponding increase in number of cures. The high dose of endostatin virus was much less toxic, as evidenced by fewer negative clinical signs. These animals were only given one dose, monitored for clinical signs, and measured for tumor volume. A log lower dose of endostatin virus was administered in 3 doses (2 days apart) to tumor-bearing mice, which also resulted in marked antitumor efficacy and survival. The implications of effective low doses of oncolytic virus involve the lowering of manufacturing costs and greater ease of production, which are often limiting factors in the development of viral vectors for various treatments.

As shown with the targeting constructs, the tumor cell environment, i.e., liver versus subcutaneous, is quite different. This could dramatically alter the dependence of a tumor cell on supporting endothelial cells (Fukumura, Yuan et al. 1997). In the liver there are already a multitude of endothelial sinusoidal cells that may supply nutrients to at least small tumors. In subcutaneous tumors, both the tumor and supporting endothelial cells within the tumors grow quite rapidly and therefore might be more sensitive to antiangiogenic therapy. In addition, it is now becoming quite well established that
endothelial cells from different organs are quite different with respect to their biological properties, including what receptors are expressed (Marti and Risau 1998). Thus it is certainly possible that endostatin could act more potently on one subset of endothelial cells (e.g., within tumors in the subcutaneous environment versus tumors within the liver).

Various anti-angiogenesis-based gene therapy strategies have been reported, including naked plasmid DNA (Blezinger, Wang et al. 1999), liposome-formulated plasmid DNA (Chen, Kumar et al. 1999), and adeno-associated viral vector (Nguyen, Wu et al. 1998). However, all these approaches have demonstrated limited efficacy as compared with the protein approach reported previously (Boehm, Folkman et al. 1997). In addition, many studies show the inability to completely abolish tumors with this therapy (Griscelli, Li et al. 1998; Dhanabal, Ramchandran et al. 1999; Sauter, Martinet et al. 2000; Joki, Machluf et al. 2001). This could be due to the tumor cell environment, efficacy of delivery and expression, and/or treatment regimens. However, our results have demonstrated that HSV-based oncolytic vectors encoding anti-angiogenesis genes are able to mediate significant oncolysis and cure animals in colorectal models of cancer. It is possible that our improved delivery system with the addition of DS to the viral inoculum contributed significantly to these results. Furthermore, this could also reflect a synergistic effect of endothelial cell killing by HSV in addition to the expression of anti-angiogenesis proteins that would result in regression of established tumor vasculature. Ultimately, there are a variety of vector improvements that could result in safer, higher, prolonged, and regulatable levels of expression of anti-angiogenesis proteins. It will be interesting to see the results of targeted vectors with regulatable expression systems in anti-angiogenesis gene therapy.

These results demonstrate the advantages of this delivery system, which allows continuous release of biologically active anti-angiogenesis proteins for the treatment of cancer. This system may overcome obstacles such as the short half-life of administered proteins, repeated administration, high doses, and cost. Additionally, with the growing data demonstrating a superadditive or synergistic effect of antiangiogenic agents in combination with other antiangiogenic agents and/or in combination with genotoxic drugs (chemotherapy) and radiation, the ultimate use of targeted anti-angiogenesis may
be in combination with other antitumor treatment modalities. This study clearly emphasizes the importance of investigating the mechanism of action of cancer therapies, such that vectors can be specifically tailored to exploit the biology of systems in order to improve the therapeutic index of proposed treatments. In this manner, targeted and efficient vectors can be engineered for the goal of improving the clinical outcome of malignant diseases.
CHAPTER 7: Discussion

7.1 Proposed Model for DS-Mediated Muscle Gene Therapy

It is well established that DS normally inhibits infection of cells by enveloped viruses (Baba, Pauwels et al. 1988; Krumbiegel, Dimitrov et al. 1992; Neyts, Snoeck et al. 1992; Ida, Kurata et al. 1994; Marchetti, Pisani et al. 1995; Dyer, Banfield et al. 1997; Fabregas, Garcia et al. 1999; Zhang, Schols et al. 1999; Hulst, van Gennip et al. 2000). However, cells normally resistant to HSV-1 infection were found to be permissive following a pretreatment with DS (Dyer, Banfield et al. 1997). These cells, termed sog9 cells, do not display any cell surface glycosaminoglycans, molecules important for the initial attachment of HSV to cells. It was suggested that DS binds to sog9 cells in a saturable, reversible manner and tethers the virion to the cell surface.

Gene transfer to skeletal muscles has been hampered in part due to the inability of current generation vectors to infect a significant number of cells (Acsadi, Dickson et al. 1991; Karpati and Acsadi 1993; Smith, Mehaffey et al. 1993; Acsadi, Jani et al. 1994; Huard, Feero et al. 1996). It was proposed by Huard and colleagues that the basal lamina served as a physical barrier to viral infection, and therefore infection could only be achieved by damaging this matrix (Acsadi, Dickson et al. 1991; Karpati and Acsadi 1993; Smith, Mehaffey et al. 1993; Acsadi, Jani et al. 1994; Huard, Feero et al. 1996).

We have demonstrated that maturation-dependent changes in HS on the myofiber cell surface reduce the ability of HSV to infect these cells. Pretreatment of mature myofibers with collagenase type IV, which enzymatically disrupts the basal lamina, resulted in only minor increases in levels of myofiber infection. However, myofiber susceptibility to HSV infection was restored following a pretreatment with DS. Our studies have shown that this interaction is saturable and reversible, requiring a specific low concentration of DS (10 µg/ml) in order to achieve stimulation of infection.

Similar to the case of sog9 cells, DS functions essentially as a substitute for cell surface HS that confers type-specific infection. The failure of DS to stimulate HSV-2 infection is likely due to the differences in glycoproteins responsible for viral attachment. It is clear that DS stimulation is mediated at least in part by gB-1, and gB-2 is inactive in
this capacity when it is present in intact HSV-1 virion (Dyer, Banfield et al. 1997). The differences in the structures of gB-1 and gB-2 may further alter their ability to adsorb to sulfated polyanions such as DS. Finally, gC-2 has been found to reduce DS-mediated infection either by interfering with the binding of the virion to DS at the cell surface, or by directly impeding the interaction of gB-2 with DS.

Our data also indicate that stimulation of myofiber infection by DS was mediated specifically by DS; soluble HS did not enhance infection. This is interesting, because HS is a natural cell surface receptor for the virus. Several cell types, including macrophages and endothelial cells, express scavenger receptors capable of binding various polyanionic ligands (Krieger 1992; Krieger, Acton et al. 1993). In particular, scavenger receptors present on the surface of murine macrophages have broad ligand binding specificity and can bind DS and fucoidan but not HS. Although the ligands for these receptors are all polyanions, not all polyanions can function as ligands. It is possible, therefore, that mature skeletal muscle possesses a receptor with a binding specificity similar to that of macrophage scavenger receptors, and this may account for our observation that cells pretreated with DS retain enhanced susceptibility to HSV-1 even after the soluble DS is removed.

In light of these results, how is AAV able to efficiently infect mature skeletal muscle? Although AAV has been shown to use HS as a co-receptor to gain entry to cells, it may not have as stringent a requirement for HS moieties as does HSV to infect cells efficiently. Furthermore, the relatively small size of AAV may allow it to penetrate the basal lamina pores better to engage its cognate receptors.

Our study shows that the basal lamina is not an absolute block to infection. DS is able to stimulate infection of mature skeletal muscle without destroying any component of the cell architecture. This discovery is important because it is non-destructive, non-toxic, and represents an approach for targeted expression of HSV vectors to muscle fibers by direct injection. It also lends further support for this additional type-specific phenotype, which involves the interaction of virions with host cell surfaces devoid of glycosaminoglycans. This interaction is only evident following the exposure of host cells to long chains of the GAG analog DS. In this manner, studies involving DS have not only led to an increased understanding of viral entry and their host biological systems,
but they have enabled us to exploit this knowledge to develop appropriate therapies for
disease (Figure 7.1).
Figure 7.1 Proposed model for DS-mediated infection of mature myofibers.

In the absence of cell-surface HS, DS is able to mediate infection of mature myofibers. On the other hand, AAV is able to efficiently infect mature myofibers because its small size allows it to interact effectively with its protein receptor for productive infection. The clear gaps seen in the basal lamina depict the basal laminar pores.
7.2 **HSV Vectors for Cancer Therapy**

The discovery of the genetic origin of numerous human diseases created the molecular basis for gene therapy. Ongoing studies of human viruses have contributed to the development of novel viral therapies that limit tumor growth, destroy existing tumors, and in some instances increase survival in animal models of disease.

HSV vectors are a recent addition to the arsenal of viral therapy, and clinical trials performed in the past year have shown human safety following direct intratumoral injection for the treatment of brain tumors. In future studies, these vectors can serve as a platform for delivering additional genetic material that may be required to enhance the efficacy of vectors. The large genome of HSV should allow for the addition of multiple genes, including cytokines, chemokines, antiangiogenic factors, or other ligands and toxins. Moreover, improvements in vector design that increase the efficiency of expression, that increase the precision of targeting, and that reduce toxicity should also improve response rates.

The demonstration that HSV is synergistic with radiation in certain animal models is encouraging, and will facilitate the development of improved treatment modalities for brain tumors and other cancers for which current therapies are inadequate. Each disease poses somewhat different challenges in terms of the choice of vector, route of delivery, and dosing. Despite these considerable challenges, the high incidence and life-threatening aspects of brain tumors and other cancers will ensure that viral-based therapy will continue to be developed and tested aggressively to determine whether it can provide statistically significant clinical benefit.

Another property of replicating viral vectors, including HSV, is that their effectiveness is enhanced when administered along with radiation or chemotherapy. In an effort to evaluate which combination of viral therapy may be more effective, experiments were carried out in which viral therapy was administered with and without these treatments. In one such study of human SQ-20B derived tumors established in mice, Weichselbaum and colleagues showed that fractionated irradiation approximating the doses applied in clinically relevant protocols was synergistic with virus and resulted in tumor regression and in some instances, cures (Weichselbaum, Hallahan et al. 1994;
Advani, Chmura et al. 1997; Advani, Chung et al. 1999). Virally treated tumors were irradiated with 400 cGy daily to a total dose of 3200 cGy. A 3-fold reduction in tumor volume with this combination treatment shows the potential efficacy of combining irradiation with genetically engineered HSV in future clinical protocols. Initial data suggest that radiation induces genes that alter transcription in the irradiated cell. This may in effect complement the attenuating defects in the viral vectors. It appears that damaged cells provide a highly active environment for viral replication, which results in the production of more virus in situ, and consequently, a greater therapeutic benefit.

Additionally, a combination of chemotherapy and viral administration has recently been shown to improve the antitumor efficacy of G207, although the therapeutic effects in vivo were shown to be independent (Chahlavi, Todo et al. 1999). Once again, this phenomenon will require additional studies to compare the benefits of viral therapy alone versus combination therapy in different tumor types.

New developments have also been made with regard to enhancing the immune response to malignant gliomas since the oncolytic effect of replication-competent HSV depends both on cell destruction by the virus and an immune response to the tumor cells. The goal is to stimulate recognition of tumor cells by the host’s immune system and to activate tumor antigen-specific cellular immunity. Recently, it was shown that regulation of the immune response does contribute to survival of glioma-bearing animals (Andreansky, He et al. 1998). By these studies, a recombinant HSV expressing IL-4 prolonged survival and was associated with infiltration of the tumor by macrophages, CD4+ and CD8+ T cells. In contrast, recombinant HSV expressing IL-10, which downregulates the immune response, led to survival no different than that seen with saline-treated controls. In a recent report, a replication-competent HSV vector expressing IL-12 was found to produce a significant survival benefit in A/J mice implanted with the syngeneic Neuro-2a clone of C1300 neuroblastoma tumor cells. In these experiments, the oncolytic effects of the vector were enhanced by IL-12-induced immunologic effects mediated by helper T cells of subset type 1 (Parker, Gillespie et al. 2000). Thus, cytokines expressed from genes encoded in the viral genome can enhance antitumor efficacy and may represent another important adjunct to tumor therapy using genetically engineered HSV.
7.3 **Dextran Sulfate and the Clinic**

Natural and synthetic sulfated polysaccharides have been tested for their prophylactic properties, and some synthetic compounds have been tested for tolerance when administered to humans (Schaeffer and Krylov 2000). Dextran sulfate, in particular, has surfaced in a therapeutic light most recently as a potential drug to treat acquired immune deficiency disease syndrome (AIDS). A number of publications have shown the anti-HIV activity of DS (Baba, Pauwels et al. 1988; Baba, Snoeck et al. 1988; Lederman, Gulick et al. 1989; Ida, Kurata et al. 1994). *In vitro* results have indicated great promise for the use of synthetic polysaccharides in AIDS therapy. However, ongoing human clinical studies have failed to reciprocate the promise of a new weapon in the fight against HIV (Abrams, Pettinelli et al. 1989; Lorentsen, Hendrix et al. 1989; Flexner, Braditch-Crovo et al. 1991; Hiebert, Wice et al. 1999).

Despite the need to demonstrate a beneficial effect of DS for the treatment of symptomatic HIV infection, these studies have yielded a great deal of toxicity data. Intravenous delivery of 900 – 5400 mg/day of DS (for one week) was safe (Abrams, Pettinelli et al. 1989). However, continuous i.v. infusion of the maximally tolerated dose (MTD) of DS for greater than 14 days was toxic (Flexner, Braditch-Crovo et al. 1991). Results indicated that the patients showed profound but reversible thrombocytopenia (Flexner, Braditch-Crovo et al. 1991).

Although the MTD has been determined for DS in humans, further studies are required to assess the toxicity of DS with respect to particular dosing regimens. The data in previous chapters have outlined a treatment regimen that is safe within the confines of existing safety data in humans. Since its use in the clinic has already been approved, DS should be considered in existing experimental viral vector protocols for the treatment of cancer.

7.4 **Mechanism of Action of Oncolytic HSV: Anti-angiogenesis**

Systemic administration of oncolytic vectors for targeted cancer therapy involves overcoming a number of hurdles in order to achieve effective antitumor efficacy. Firstly, the vectors used must be safe such that viremia and uncontrolled infection are not consequences to treatment. In addition, large quantities of active viral particles must
reach the tumor site for oncolysis. Since one treatment is not likely to result in complete eradication of all tumor cells, the vector must be amenable to repeated administration. Therefore pre-existing immunity must not preclude fractionated delivery of the vector. HSV vectors are an attractive choice because they satisfy all these requirements.

In both solid and metastatic models of cancer, we found systemic delivery of DS-coated HSV to be effective at delaying tumor growth and, in 25% of the animals, eradicating tumors. Furthermore, administration of a log lower viral dose ($10^5$ PFU) along with DS was also sufficient to mediate significant antitumor efficacy. The results, although remarkable, were puzzling. In treatment models that involve direct injection of oncolytic vectors, higher doses ($10^7$ PFU) are routinely required to achieve similar results. How then could such low doses of virus eradicate tumors in a systemic model of cancer?

Uncovering the mechanism behind the marked oncolysis seen with systemically delivered HSV is important from both a biological and practical standpoint. In the first instance, it offers us a better understanding of animal systems and viral vectors. In the latter instance, it enables us to utilize this knowledge to improve upon the safety and efficacy of engineered vectors. With this in mind, we investigated the role of DS in improving i.v. delivery of oncolytic HSV and the subsequent mode of tumor kill.

DS is typically an inhibitor of viral infection. However, in cases where the host cell lacks cell surface GAGs, DS acts as an artificial receptor to tether the virus to the cell surface such that it can engage secondary protein receptors. A number of publications have shown that this function of DS requires a number of conditions to be met. To begin with, the host cell must be relatively devoid of cell surface GAGs. Furthermore, a specific concentration of DS (typically very low i.e. 10 μg/ml) is required for optimum action. Therefore, in this case, DS function does not improve in a dose-dependent manner. Next, the sulfate groups on DS are essential, and hence dextran is not able to mediate infection of such cells. In our animal models of cancer, we found CT26 cells to be infectable, thereby indicating that they display cell surface GAGs. In addition, DS improved viral antitumor efficacy in a dose-dependent manner, and dextran was shown to function as well as DS. Therefore, we examined other known properties of DS for their potential to improve the therapeutic index of oncolytic HSV.
DS has been used in the clinic as a drug carrier as well as a volume expander. Pharmacokinetic analyses of DS-coated HSV indicated that DS increased the bioavailability of active virus in circulation over time. In this manner, it likely served to protect infectious viral particles and released them over time for tumor kill. Moreover, tumor vasculature is characteristically leaky. Therefore DS activity as a volume expander would result in a temporary increase in blood pressure, which may have allowed for selective extravasation of viral particles to tumor. This would also account for the passive targeting of HSV to tumor seen in radioactive distribution studies. The combination of all the factors illustrates how DS is able to alter the hemodynamic properties of oncolytic HSV in favor of marked tumor destruction.

The data so far could explain how the addition of DS resulted in relatively more virus at the portals to tumor. However, the mode of tumor kill by oncolytic HSV still needed to be resolved. Direct injections of higher doses of HSV into tumor were necessary to show similar antitumor efficacy and cures. Yet, radioactive distribution experiments of systemically delivered HSV indicated that relatively little virus reached the tumor (approximately 1000 PFU). This suggested that the major targets were not likely the tumor cells, but rather the tumor endothelial cells. This was further supported by marked peripheral degeneration of i.v. treated tumors. In addition, CD31 immunohistochemistry coupled with the delivery of GFP-expressing HSV showed that tumor endothelial cells were infected by HSV and tumor infection was mainly localized to the periphery. Although, endothelial cell apoptosis may have a larger role in our treatment model, HSV-mediated tumor cell kill likely involves a combination of direct oncolysis, secretion of anti-angiogenesis factors by target cells, and immune modulation (Figure 7.2).

If tumor endothelial cells were the major targets, improving endothelial cell killing by targeting HSV directly to the vasculature or by engineering HSV to carry anti-angiogenesis genes should increase the potency of therapy. In both of these cases, antitumor efficacy was significantly improved, with a resultant increase in animal survival. Not only were we able to develop a working model of how systemically delivered HSV results in antitumor efficacy, but we were able to take this knowledge a step further to develop HSV vectors with an improved therapeutic index.
Acquired drug resistance is a major problem in the treatment of cancer. Most of the annual deaths from cancer follow the development of resistance to chemotherapy (Li, Schneider et al. 1993). The emergence of resistance depends in part on the genetic instability, heterogeneity and high mutational rate of tumor cells (Li, Schneider et al. 1993). In contrast, endothelial cells are genetically stable, homogenous and have a low mutational rate. Therefore, antiangiogenic therapy directed against a tumor’s endothelial cells should, in principle, induce little or no drug resistance.

The results of this study illustrate the powerful control exerted by the vascular endothelial cell population over the tumor cell population. It remains to be seen whether a lack of drug resistance will be a general property of angiogenesis inhibitors. It may be predicted that those angiogenesis inhibitors that specifically or selectively target vascular endothelial cells will be less likely to induce resistance than inhibitors which target a tumor-derived mediator of angiogenesis. As an example of the latter, tumors that produce basic fibroblast growth factor as their predominant angiogenic mediator, may initially respond to interferon alpha-induced suppression of bFGF production by tumor cells (Singh, Gutman et al. 1995). However, as cells arise which produce other angiogenic factors, the tumor itself may acquire resistance to an angiogenesis inhibitor as specific as endostatin, should they generate a degrading enzyme, but to date this has not been observed. It should also be emphasized that antiangiogenic therapy when combined with toxic agents and radiotherapy produces enhanced antitumor efficacy in tumor bearing animals (Shepherd 1997; Mauceri, Hanna et al. 1998). In the future, antiangiogenic therapy can be used alongside conventional anticancer therapies such as surgery, chemotherapy, radiotherapy or immunotherapy. Therefore angiogenesis inhibitors that do not induce drug resistance may be valuable for longterm maintenance therapy.
Dextran sulfate – coated virus allows for HSV accumulation at tumor vascular portals by a number of mechanisms: passive targeting, increasing the bioavailability of the virus in circulation, and altering the hemodynamic properties in favor of viral extravasation at the site of the tumor. Once the oncolytic virus has reached its target region, antitumor efficacy can be mediated by a combination of direct oncolysis, secretion of anti-angiogenesis factors, and more importantly, endothelial cell apoptosis.
7.5 **Future Potential and Direction of Gene Therapy**

Fundamental research into the mechanisms of cancer and the development of molecular biology tools are crucial for the success of the treatments in the future. All currently used chemotherapeutic agents have their maximal dose at the time of administration and thereafter decline in concentration. The possibility of developing an anticancer therapy whose activity can increase with time while retaining tumor-specificity is a new and uncharted area of cancer therapy. It is well known that conditionally-replicating vectors based on HSV can be applied safely even for direct inoculation into the most HSV sensitive organ, the brain. This reassurance opens the possibility of exploring HSV vectors for the treatment of many of the common cancers afflicting humankind. Moreover, preclinical studies thus far suggest that they are both safe and efficacious following intravascular delivery and can be effectively used in conjunction with chemotherapy, radiotherapy, and immunotherapy.

The potential applications of gene therapy in the treatment of disease currently are limited by the inability to adequately deliver genes to target tissues and to maintain their expression. Because different tissues have diverse biological properties, and since even the most developed of viral vector systems has its limitations, it is unrealistic to imagine that one virus will eventually emerge as the most suitable vector for all gene therapy applications. Rather, successful gene therapy likely lies with the ability to create highly specialized “designer” vectors that safely incorporate specific advantageous characteristics from several viruses to address specific features of a disease and the target tissue for treatment.

Gene therapy represents one of the most important developments in oncology. However, before this can be realized as a standard treatment, the technical problems of gene delivery and safety must be overcome. Many improvements in vector technology, though, are likely to be preceded by advances in understanding of the biology of specific tissues and cells with regard to gene delivery and their ability to maintain transgene expression. These factors include such considerations as cellular turnover, surface receptors, antigen presentation, tissue-specific gene expression, and responsiveness to specific cytokines and hormones. As more becomes known about specific cellular
requirements for effective gene transfer and expression, "designer" vectors can be engineered specifically for a particular cell type. Combination strategies and consequently the specific tailoring of different methods to attack disease will prove to be the top choice in standard protocols of care in medicine. The success of combination therapies underlines the fact that the different approaches can be synergistic and that attacking a disease with different weapons could be beneficial.

Future research should also be focused in modifying viral vectors to reduce toxicity and immunogenicity, increasing the transduction efficiency of nonviral vectors, enhancing vector targeting and specificity, regulating gene expression, and identifying synergies between gene-based agents and other cancer therapeutics. The future improvement of present viruses as well as the use of new viral vectors will likely expand the applicability and efficacy of gene therapy. Toxic effects associated with the use of viral vectors for gene transfer in humans can likely be overcome by modifying vector structure to eliminate expression of endogenous viral genes, improving vector targeting and using immunomodulators to reduce the immune response against the vector.

One of the more important aspects of gene therapy continues to be the specificity of therapeutic gene expression. A number of studies have shown that viral vectors can be targeted to specific cell types after attachment of ligands to the viral capsid or manipulation of envelope genes to express chimeric proteins that consists of the envelope with a cell-specific ligand (Sosnowski, Gu et al. 1999; Fisher, Stallwood et al. 2001; Maxwell, Chapman et al. 2001; Muller, Nahde et al. 2001). The next level of specificity can be generated by using tissue or cell-specific promoters. The duration of therapeutic gene expression is another important determinant of the effectiveness of the therapy.

Safety of viral vectors is, at present, one of the most critical aspects of human gene therapy. The recent treatment-related death of a young man due to a systemic inflammatory response to the therapeutic adenoviral construct has raised major concern on the safety issue (Hollon 2000). The recombinant DNA Advisory Committee, in examining the event, presented a list of problems linked to adenovirus safety. Standards for adenovirus titration, the measurement of transgene expression, and a better assessment of immune status before and after treatment are needed. The Committee also indicated that studies of vector biodistribution must be performed and better quality
control for the integrity of vector DNA should be obtained (Hollon 2000). Thus safety issues should be a focus for all novel biological therapies.

Results from current preclinical and clinical studies suggest that gene therapy approaches eventually will succeed in providing clinically efficacious treatment for at least some human diseases. Exactly which diseases will be the most effectively treated by gene therapy remains unclear. As vector technology improves, the ability to treat human disease should also improve. Although much research needs to be done, the possibility of specific gene targeting with a high therapeutic index makes this area of gene therapy a promising one for future investigations.

7.6 Bench to Bedside

During the last two decades there have been substantial gains in our comprehension of the pathogenesis of cancer. Many of these insights have occurred at a molecular level and include a better understanding of the multistep nature of carcinogenesis as well as the pivotal role of tumor suppressor and transforming oncogenes in this process. The axiom underlying the basic research is that a better appreciation of transforming events will lead to a new generation of rationally designed pharmaceuticals – including a class of compounds which will act primarily at the genetic level.

Gene therapy has applications across many fields of medicine, particularly the treatment of cancer. The heterogeneity of tumors makes it unlikely that a single principal approach will prove effective for all tumors. Future approaches will probably rely on combinations of immune adoptive therapy, molecular chemotherapy and/or prodrug conversion in addition to protective treatments of normal cells (drug resistance).

Supplying the tools will benefit not only patients with cancer but will also influence treatment of other acquired and hereditary diseases. In this manner, new approaches are constantly being applied to old dilemmas, but they themselves will raise new challenges as well. Moreover, it will fuel further inquiry into the molecular basis of human disease.

Although the first clinical protocols in gene therapy began just over 10 years ago, progress has been rapid, and important observations have emerged from ongoing trials.
Applying novel therapies to human disease will require the cooperation of physicians and investigators in the basic sciences to develop safe and effective systems for clinical use. It is anticipated that practicing clinicians will need to incorporate this new information into their daily clinical practice in the near future.
REFERENCES


APPENDIX 1

Chemical Reagents and Laboratory Supplies

Alcohol swabs
Acetic acid
Acetone
Acyclovir
Agarose
Ammonium acetate
Ampicillin
AntiFade
AquaPerm
Bovine serum albumin (BSA)
Bromophenol blue
Chloroform
Chondroitin ABC lyase
Chondroitin sulfate C
Collagenase type I
Collagenase type IV
DEAE-Sephadex
Dextran sulfate
Dextrose
Dimethyl sulfoxide (DMSO)
Dulbecco’s Modified Eagle Media (DMEM)
DMEM without glutamine, methionine, cysteine
EDTA
Ethidium bromide
Fetal Bovine Serum (FBS)
Filters
Glutaraldehyde

BDH
Fisher
Fisher
AltiMed
Canadian Life Technologies
Fisher
Sigma
Molecular Probes
Immunon
Roche
VWR
Fisher
Seikagaku
Sigma
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Pharmacia
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Canadian Life Technologies
ICN
Fisher
Sigma
Canadian Life Technologies
Millipore
Sigma
Glycerol
Heparitinase
Horse serum (HS)
Human gamma globulin
Hydrochloric acid (HCl)
Isopentane
Ketamine
L-glutamine
Matrigel
Methanol
Methylene blue
OCT Tissue-Tek
Paraformaldehyde
Paraformaldehyde (16% EM-grade)
Penicillin/Streptomycin
Phosphate buffered saline (PBS)
Polyethylene glycol 8000 (PEG)
Potassium ferricyanide
Potassium ferrocyanide
Potassium Phosphate (monobasic)
Potassium Phosphate (dibasic)
Protease
Rhopun
RPMI medium 1640
Saponin
Scintillation cocktails (Ready Safe)
$^{35}$S-methionine
Sodium acetate
Sodium Chloride (NaCl)
Sodium deoxycholate
Sodium hydroxide
Fisher
Seikagaku
Canadian Life Technologies
ICN
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Biomed-MTC
Canadian Life Technologies
Collaborative Biomedical
Fisher
Sigma
Sakura Finetek
Fisher
Canemco
Canadian Life Technologies
Canadian Life Technologies
BDH
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Bayer
Canadian Life Technologies
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Beckman
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Sigma
Fisher
Sodium Phosphate (monobasic)  Mallinckrodt
Sodium Phosphate (dibasic)  Fisher
35S-sulfate  ICN
Sucrose  Fisher
Surgical instruments  VWR
Syringes  BDH
Tissue culture flasks and dishes  Canadian Life Technologies
Tris  Canadian Life Technologies
TSK DEAE-35W column  Beckman
Triton X-100  Sigma
Trypsin  Canadian Life Technologies
Ultracentrifuge rotors and tubes  Beckman
X-gal  Canadian Life Technologies
Zwittergent 3-12  Calbiochem
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