ANN-MARIE MÄÄTTÄ

Development of Gene and Virotherapy Against Non-Small Cell Lung Cancer

Doctoral dissertation

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Lung cancer is the leading cause of cancer deaths worldwide. The delay in diagnosis, often only when there is already invasive and metastatic disease, is one reason for very bleak prognosis; less than 15% of patients live more than 5 years. Current treatments (chemotherapy, radiotherapy and surgery) remain mostly palliative for most patients and therefore there is an urgent need to develop more efficient and better targeted therapies against lung cancer. The aim of this study was to evaluate two anticancer modalities to combat non-small cell lung cancer (NSCLC).

Herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) is the oldest and most extensively studied cytotoxic gene therapy modality, where the transferred thymidine kinase gene product converts the normally non-toxic compound GCV into its toxic form, leading to cell death. The HSV-TK/GCV anticancer effect was evaluated here in NSCLC cells in vivo and in vitro. The adenoviral gene transfer proved to be efficient in vitro and in vivo and led to powerful tumor cell eradication in cell culture studies and in subcutaneous tumor models after GCV exposure. The presence of a positive bystander-effect in the tested lung cancer cell lines was shown to be an essential facet in achieving an efficient response to therapy. However, more controlled regulation of the therapeutic gene could further improve the safety and also the efficacy. We targeted the therapeutic gene (HSV-TK) transcriptionally with human hexokinase II (hHKII) promoter. The hexokinase II is known to be overexpressed in cancer cells and in this work we confirmed that the short fragment of hHKII promoter was highly efficient in cancer cells but showed virtually no transgene expression in human primary cells.

Virotherapy is based on the conditionally replicative properties of the used viruses, meaning that viruses replicate only in (and lyse) cancer cells inherently or after genetical manipulation. The Semliki Forest virus (SFV) virotherapy based on avirulent strain A7(74), when administered locally was demonstrated to be very efficient at killing cancer cells in vitro and proved to be a potent anticancer agent in immunocompromised subcutaneous and orthotopic lung tumor models when administered locally. However, the lack of response achieved with the systemic route in nude mouse tumor models and in locally administered immunocompetent tumor model (rat glioma model) demonstrates that the presence of immune system further complicates the situation; only a limited amount of the virus is able to gain access to the tumor, components of the immune system neutralize the virus and therefore sufficiently powerful replication cannot be achieved. Even immune suppression with dexamethasone did not improve the response to virotherapy in immunocompetent animals.

In conclusion, these pre-clinical studies demonstrate that HSV-TK/GCV gene therapy and SFV A7 (74) virotherapy are safe and efficient anticancer methods against NSCLC. However, as shown in virotherapy studies, the immune system plays a critical role in virus-mediated gene therapy forms and further studies are needed to resolve how to circumvent (or exploit) the intrinsic barriers that are encountered in immunocompetent animals to enhance the response to these novel treatments.

National Library of Medicine Classification: WF 658, QZ 52, QZ 266, QW 168.5.A7
Medical Subject Headings: lung neoplasms/therapy; carcinoma, non-small-cell lung/therapy; gene therapy; gene targeting; genetic vectors; simplexvirus; thymidine kinase; ganciclovir; antineoplastic agents; oncolytic virotherapy; oncolytic viruses; semliki forest virus; cells, cultured; cell line, tumor; disease models, animal; glioma; immune system
To Saku, Elmo & Emmi
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Kuopio, September 2007

Ann-Marie Määttä
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>adeno associated virus</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell leukemia/lymphoma 2 genes</td>
</tr>
<tr>
<td>CDKs</td>
<td>cyclin-dependent protein kinases</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus immediate early promoter</td>
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<tr>
<td>CPA</td>
<td>cyclophosphamide</td>
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<tr>
<td>CRAd</td>
<td>conditionally replicating adenovirus</td>
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<tr>
<td>CT</td>
<td>computerized tomography</td>
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<td>CTL</td>
<td>cytotoxic T lymphocytes</td>
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<tr>
<td>CVF</td>
<td>cobra venom factor</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>eIF-2α</td>
<td>α subunit of eukaryotic polypeptide chain initiation factor 2</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>GCV</td>
<td>ganciclovir</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GBM</td>
<td>glioblastoma multiform</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
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<tr>
<td>GVAX</td>
<td>GM-CSF gene modified tumor vaccine</td>
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<tr>
<td>HER-2</td>
<td>human endothelial growth factor two</td>
</tr>
<tr>
<td>hEF1α</td>
<td>human elongation factor 1 alpha promoter</td>
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<tr>
<td>hHKII</td>
<td>human hexokinase II promoter</td>
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<tr>
<td>HSV-1</td>
<td>herpes simplex virus type I</td>
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<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
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<tr>
<td>IARC</td>
<td>international agency for research on cancer</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Mdm2</td>
<td>murine double minute oncogene</td>
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<td>MV</td>
<td>measles virus</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>p14ARF</td>
<td>alternate reading frame product of the INK4 gene</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>pfu</td>
<td>plaque forming unit</td>
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<tr>
<td>PKR</td>
<td>double stranded RNA-dependent protein kinase</td>
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<tr>
<td>PP1α</td>
<td>protein phosphatase 1α</td>
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<tr>
<td>RR</td>
<td>ribonucleotide reductase</td>
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<tr>
<td>SCLC</td>
<td>small cell lung cancer</td>
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<td>SFV</td>
<td>Semliki Forest virus</td>
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<td>SLAM</td>
<td>single lymphocyte activation molecule</td>
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<tr>
<td>TGF-α</td>
<td>transforming growth factor alpha</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TK</td>
<td>thymidine kinase</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis inducing ligand</td>
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<tr>
<td>TU</td>
<td>transducing unit</td>
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<tr>
<td>VA-RNA</td>
<td>Virus-associated RNA</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
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<tr>
<td>VSV-G</td>
<td>G protein from vesicular stomatitis virus</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WPRE</td>
<td>woodchuck hepatitis virus post-transcriptional element</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by the corresponding Roman numerals.


1 INTRODUCTION

Cancer is a major burden worldwide, the incidence being high: annually 10.1 million new cases, 6.2 million deaths and 22.4 million people living with cancer (WHO and IARC, 2003). The heterogenic nature of cancer makes it more difficult to treat and therefore responses to current treatments vary from case to case. Non-small cell lung cancer (NSCLC) is one of the most prevalent cancer types and the number one cause of cancer deaths (overall survival < 15%). The highly metastatic nature of lung cancer and the delay in diagnosis, because of its asymptomatic early stage, are the main reasons for the poor responses achieved with today’s conventional treatment modalities. Especially in the case of highly malignant cancers, better targeted therapies that would reach also the metastases are needed.

The achievements in molecular biology and gene technology have led to an extensive field of research called gene therapy. This rapidly growing area has yielded a number of approaches against cancer, all of them focusing on providing more precise and localized anticancer actions and this way preventing, or at least reducing, the unwanted side-effects in normal tissues. Several modalities have already entered into clinical phase and the first commercial gene medicine against cancer has been approved. Essential issues such as safety and efficacy have been further enhanced by targeting the therapy transcriptionally (promoters for tumor-specific transgene expression) or transductionally (modification of the gene transfer agent for enhanced affinity to tumor cells). Nonetheless, there is one fundamental problem in gene therapy with replication-deficient viruses, i.e. the lack of gene transfer efficacy in vivo, which leads to an inefficient response to therapy. One solution is to use replication competent viruses that can multiply in malignant cells and lyse them as the replication cycle is completed. The viruses used in virotherapy selectively replicate in malignant cells and in this way enhance the therapeutic outcome and the safety, providing also the possibility to achieve systemic antitumor responses that would be desirable when treating metastatic cancers such as lung cancer.

In this work, two different approaches were evaluated against NSCLC; classical suicide gene therapy (HSV-TK/GCV) and virotherapy based on replication competent avirulent Semliki Forest virus. We focused on essential questions such as safety and efficacy that need to be carefully evaluated before any novel treatment strategy can enter into clinical studies.
2 REVIEW OF THE LITERATURE

2.1 LUNG CANCER

2.1.1 EPIDEMIOLOGY AND ETIOLOGY

Lung cancer has been the most common cancer since 1985 and this disease is also one of the leading causes of cancer deaths worldwide (Parkin et al., 2005). It has been estimated that in the U.S. lung and bronchial cancers will be the primary cause of cancer deaths in the year 2007 (160,390 deaths) (Jemal et al., 2007). In Finland the incidence of lung cancer among men has been declining but on the contrary among women it has risen (1955-2004). It is the second most common cancer in men (1537 new cases in 2004) and third most common in women (596 new cases in 2004) and the mortality is very high in both sexes. The overall 5-year survival rate for all lung cancer patients is less than 15 percent (Finnish Cancer Register, www.cancerregistry.fi).

Lung cancer is one of the few cancers where a strong causal connection to environmental factors (i.e. smoking) has been shown; especially the duration of smoking has proved to have a strong effect on lung cancer mortality (Flanders et al., 2003). It has even been speculated (Mattson et al., 1999) that if all smokers were able to stop smoking, then lung cancer would almost vanish in a short period of time and this would mean around 1.2 million fewer annual cancer deaths worldwide. In the case of lung cancer there are also other environmental factors like exposures to radon gas, asbestos, silica and some metals (nickel, arsenic and cadmium) that have been shown to increase lung cancer risk. Finally, a history of lung diseases such as asthma, chronic bronchitis, pneumonia or tuberculosis can increase the risk of developing lung cancer (Cassidy et al., 2007).

Lung cancer is classified into two major groups, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter comprising 80% of the lung cancer cases (Rom et al., 2000). The group NSCLC can be further subdivided into three major histological types; squamous cell lung cancer (displays keratin formation), adenocarcinoma (forms glands that secrete mucin) and large cell lung carcinomas (are composed from undifferentiated cells that do not conform to criteria of other types) (Tyczynski et al., 2003). In Finland, the most common lung cancer type is squamous cell lung cancer (43% of all cases) with the other types occurring in more or less equal frequencies (Mattson et al., 1999).
2.1.2 PROGNOSTIC FEATURES AND CURRENT TREATMENTS FOR NSCLC

The histologically different lung cancer types differ in their clinical features and also in their responses to treatments and the prognosis. The treatment to be used has to be evaluated carefully since many factors affect the outcome of the therapy. One crucial factor to be defined is the TNM-status (T = primary tumor, N = regional lymph nodes and M = distant metastasis) of the NSCLC tumor as this influences greatly the prognosis and the treatment modality to be used (Blanchon et al., 2006; Mountain, 1997).

With the introduction of the sophisticated tools of molecular biology, lung cancer pathogenesis is becoming better understood and it appears to be more complicated than thought earlier (Rosell et al., 2004). Alterations in cell-signaling and regulatory pathways either by overexpression or down-regulation of genes are frequent events in the process leading to lung cancer. The possibilities to identify different molecular abnormalities behind NSCLC could be exploited as a prognostic factor, since even in patients with similar clinical and pathological features, the responses to treatments can still vary. The main alterations discovered are in receptor tyrosine kinases (such as the epidermal growth factor receptor, EGFR), that can affect angiogenesis pathways, apoptosis pathways and cell cycle control etc. (Molina et al., 2006). The Ras oncogene mutation has been associated with 15 - 20% of NSCLCs and particularly with 30 - 50% of lung adenocarcinomas (Rodenhuis et al., 1988). Ras oncogene (Mascaux et al., 2005) and B-cell leukemia/lymphoma 2 gene (bcl-2) (Martin et al., 2003) mutations have been linked to a positive impact on the survival, whereas deficiency in p53 tumor suppressor gene expression has been shown to have a negative impact on survival (Steels et al., 2001). Therefore, modern gene expression profiling based on microarray studies could provide useful information for classifying the tumors when evaluating the prognosis and considering the optimal treatment for the patients (Chen et al., 2007).

The first line treatment for NSCLC is radical surgery that improves the prognosis of the patient (Adebonojo et al., 1999; Mountain, 1997). Due to the fact that lung cancer is asymptomatic in its early stages and it is usually only diagnosed in the late metastatic phase, only 25 % of the patients are potential candidates for radical surgery. Usually patients are treated with chemotherapy, radiotherapy or with combination therapies, but still the prognosis is not significantly improving and the treatments remain mostly palliative (Belani and Langer, 2002; Stinchcombe et al., 2006; Vokes et al., 2002). Even though there have been some improvements in the survival rates of cancer patients, in several malignancies the responses to conventional therapies (radiotherapy and chemotherapy) are still poor. Also, the patient’s quality of life is
severely affected since there are many major side effects associated with chemotherapy (e.g. toxicity and increased mortality) and radiotherapy (e.g. infertility) (Byrne, 1999; Nemunaitis, 2003; Wei et al., 2005). Lung cancer continues to be a deadly disease, curable only in its early stages with radical surgery. Therefore, it seems that the utility of conventional therapies has reached its plateau since the response rates in advanced NSCLC are dismal (Azim and Ganti, 2006) and the future of patients with locally advanced or metastatic NSCLC looks hopelessly bleak.

The late diagnosis and the low efficacy of today’s treatments provides the impetus to test new therapy modalities to obtain better responses to improve the quality of life and to increase the survival time of the NSCLC patient. Modern molecular biological techniques have extended and expanded the research knowledge about prognostic factors so that these now include the proteins and genes involved in cancer development and this information can then be further exploited in developing new therapies (Mascaux et al., 2005). These advances include novel agents such as small molecular inhibitors of tyrosine kinases, monoclonal antibodies and direct inhibitors of proteins involved in lung cancer proliferation (Bröker and Giaccone, 2002; Molina et al., 2006). A wide range of molecular targeted agents are being investigated to be used alone or to be combined with conventional modalities (Hoang et al., 2002). However, the complicated molecular events behind NSCLC and their relationship with novel targeted drugs make the responses still quite difficult to predict (Spicer and Harper, 2005).

The burgeoning knowledge of genetics and innovative applications of biotechnology provide new weapons to combat these serious diseases like NSCLC. It has been anticipated that new breakthroughs could be based on different forms of gene therapy (Hege and Carbone, 2003) and virotherapy (Cross and Burmester, 2006; Ring, 2002).

2.2 GENE THERAPY FOR CANCER

2.2.1 CONVENTIONAL GENE THERAPY

Uncontrolled cell proliferation is the hallmark of cancer (Sherr, 1996). Tumor development in humans is a multistep process, where cells become malignant through alterations in the regulatory pathways that govern normal cell proliferation and homeostasis (Hanahan and Weinberg, 2000). There are six essential functional abnormalities in cancer formation (Figure 1.) that occur because of mutations in essential growth arrest and guardian genes. Gene therapy utilizes the advanced knowledge of the genes involved in cell transformation and targets these
genes to cure or destroy the malignant cells (Gottesman, 2003). Conventional gene therapy uses replication deficient vectors expressing a transgene to evoke the anticancer effect (Everts and van der Poel, 2005). These can be divided into four mainstream strategies based on the therapeutic gene involved: corrective gene therapy, anti-angiogenic gene therapy, immunotherapy and cytotoxic/suicide -gene therapy.

Figure 1. The typical characteristics of cancer. Tumor genesis is a multi-phased process which involves also genetic alterations that drive the progressive transformation of normal human cells into highly malignant cells (based on Hanahan and Weinberg, 2000).

The most direct application of cancer gene therapy is to provide functional genes to cancer cells i.e. to correct their defects (proapoptotic gene like Bax, tumor necrosis factor-related apoptosis inducing ligand TRAIL or tumor suppressors like p53), resulting in cell death and growth arrest (McCormick, 2001). There are several studies that have used different tumor suppressors to inhibit tumor growth in vitro and in vivo (van Beusechem et al., 2002). The provision of apoptotic inducers such as Bax (Murphy et al., 2001) and soluble TRAIL (Shi et al., 2005) has been shown achieve significant tumor growth arrest in NSCLC tumor bearing animals. p53 is probably the most widely studied tumor suppressor gene (Gottesman, 2003) since it seems to be the most common genetic alteration in human cancers (Levine et al., 1991).
A gene therapy trial (against NSCLC) utilizing p53 was published already ten years ago (Roth et al., 1996) and in 2004 the China’s State Food and Drug Ministry (SFDA) released the first approved gene therapy medicine, Gendicine – a recombinant adenovirus that contains the p53 gene (Guo and Xin, 2006). A rather similar product called INGN-201 (Lang et al., 2003) is being evaluated against several cancers already in phase III trials in Germany and the U.S.A., used either alone or as combination therapy (Journal of Gene Medicine, http://www.wiley.co.uk/genmed/clinical/).

Tumor angiogenesis is crucial for the progression of the cancer since the accelerated growth rate increases the need for a blood supply (nutrients, oxygen and growth factors) and without angiogenesis, the tumor nodules would not grow beyond a diameter of 2 - 3 mm (Carmeliet, 2005; Folkman, 1971; Risau, 1997). Genes that control angiogenesis are upregulated in many cancers and thus the inhibition of angiogenesis leads to starvation of the tumor cells and protracted tumor growth (Vile et al., 2000). Most studies have focused on inhibiting the overexpressed vascular endothelial growth factor (VEGF) or fibroblast growth factors (FGF) in tumor cells using a variety of techniques. One successful approach has been based on the use of antiangiogenic proteins such as angiotatin and endostatin to inhibit the endothelial cell proliferation leading to reduced capillary formation in tumors (Nguyen et al., 1998; Wu et al., 2003). Other promising strategies have included reducing the tumor growth by directing the antisense RNA against VEGF (Nguyen et al., 1998) or by providing a soluble VEGFR-2 that can form heterodimere complex with the wild type VEGFR-2 thus acting as a dominant negative receptor (Wu et al., 2006).

Another popular approach in the field of gene therapy has been to target the primary cancer and the metastasis with the patients’ own immune system by stimulating it with cytokines or tumor-associated antigens (Vile et al., 2000). To produce effective and long term immunity against tumor cells, the function of T-helper cells, cytotoxic T-cells and antigen presenting cells (APCs) are required (Smyth et al., 2001; Wei et al., 2005). So called GVAX (GM-CSF gene modified tumor vaccine) that contains a cytokine called granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to induce cytotoxic events in tumor cells (e.g. non-small cell lung cancer). The stimulation is mediated through dendritic cells (DCs, the most potent antigen presenting cells) (Ribas et al., 2002) and has been shown to produce anticancer immunity in pre- and clinical studies (Nemunaitis, 2003). Another fascinating approach is to generate an immune response against a known tumor antigen by engineering the patients’ blood lymphocytes to re-express high-affinity T-cell receptors that recognize these tumor antigens (Cohen et al., 2005;
Morgan et al., 2006; Zhao et al., 2006). Also, viruses without any immune stimulatory genes (replication competent or deficient) have been shown to elicit vaccine-like immune responses against tumors (Murphy et al., 2000; Toda et al., 1999).

Cytotoxic gene therapy is based on the prodrug-activating enzymes that can convert a non-toxic compound into a toxic one (Aghi et al., 2000). An excellent feature of this approach is the bystander effect that also leads to death of the non-transduced neighboring cells (Culver et al., 1992; Kurdow et al., 2002). The most extensively studied prodrug/enzyme therapy is herpes simplex virus type I thymidine kinase (HSV-TK)/ganciclovir (GCV) therapy. GCV prodrug is an acyclic nucleoside analog which is converted by the HSV-TK enzyme to the GCV-monophosphate form and further phosphorylated by cellular kinases to the triphosphate form that binds to DNA and terminates the replication (Moolten, 1986) (Figure 2.). This approach has been shown to be effective in killing several cancer types (Hasegawa et al., 1993; Ketola et al., 2004; Pulkkanen et al., 2001; Tyynelä et al., 2002) and it has also been demonstrated to prolong the survival of malignant glioma patients (Immonen et al., 2004). Furthermore, other combinations like cytosine deaminase/5-fluorocytosine have been evaluated in different tumor types (Kuriyama et al., 1999b; Miller et al., 2002).

Figure 2. Schematic presentation of the HSV-TK/GCV suicide gene therapy.

The vectors that are used to transfer the therapeutic gene play a very critical role in achieving a response to the treatment. Most studied vectors are derived from viruses since their efficiency in vivo has been shown to be much higher than their non-viral counterparts (Verma and Weitzman, 2005). The positive features for non-viral vectors are fewer toxic and immunological problems when compared to viral vectors (Somnia and Verma, 2000). The viral vectors used in conventional gene therapy lack the genes that are required for viral replication and they cannot
generate infectious virions. The most commonly used gene transfer vectors in cancer gene therapy are based on adenovirus (Kaplan, 2005; Sterman et al., 1998; Wiewrodt et al., 2003), adeno-associated virus (AAV) (Ma et al., 2002; Ponnazhagan et al., 2001; Shi et al., 2005) and retrovirus (Culver et al., 1992; Rainov, 2000; Roth et al., 1996) but the field of cancer gene therapy is evolving and new candidates for virus vectors such as lentivirus (Pellinen et al., 2004; Trono, 2000; Uch et al., 2003), Semliki Forest virus (SFV) (Loimas et al., 2000; Ren et al., 2003; Smyth et al., 2005; Yamanaka, 2004), Sindbis virus (Tseng et al., 2004) and baculovirus (Mäkelä et al., 2006; Wang et al., 2006) have been evaluated for their potency as gene transfer tools.

2.2.2 VIROTHERAPY

The low efficacy of conventional gene therapy, especially due to the limited gene transfer rate in vivo has triggered a search for novel approaches (Kirn et al., 2001; Vile et al., 2002). Virotherapy, which uses replication competent oncolytic viruses or vectors to kill tumor cells, is a promising approach for more efficient tumor destruction (Biederer et al., 2002). One important facet in this approach is to exploit the differences between malignant cells and healthy cells and find/engineer viral strains that will selectively replicate only in neoplastic cells (Mohr, 2005). The selective replication (lack of replication in normal tissues) within tumor tissue magnifies the input dose and theoretically notably increases the therapeutic impact of these agents (Bell et al., 2002; Norman and Lee, 2005; Parato et al., 2005). In addition to direct cell lysis caused by replication cycle, there are also other mechanisms that viruses can use for killing the cancer cells. They can destroy the neoplastic cells through the expression of toxic proteins, inducing both T-cell mediated immunity and inflammatory cytokine responses, or by enhancing cellular sensitivity to these effects (Kirn, 2000).

The fact that viruses replicate readily in malignant cells was recognized already at the beginning of the last century, when significant tumor regression after rabies vaccination was observed (de Pace, 1912) reviewed by (Everts and van der Poel, 2005). More recently cases were reported where cancer patients exhibited regression of Burkitt’s and Hodgkin’s lymphoma after natural measles infection (Bluming and Ziegler, 1971; Taqi et al., 1981) reviewed by (Aghi and Martuza, 2005). Already during the 1950s, clinicians had treated cancer patients with several wild-type human and animal viruses such as mumps virus, Egypti 101 virus, Sendai virus, Newcastle disease virus (NDV), influenza A, SFV, Sindbis virus, and vaccinia virus (Asada, 1974; Newman and Southam, 1954; Southam and Moore, 1952; Wheelock and Dingle, 1964). Of the above mentioned studies, the most promising was the mumps virus study, where only
mild toxicity was reported and 37 of 90 patients experienced tumor regression to more than half of the original size or complete tumor eradication occurred (Asada, 1974). There was a quiet period in the development of oncolytic therapy in the 1970s and 1980s due to toxicity, lack of selectivity and efficacy in these earlier studies and these problems raised regulatory barriers to further clinical trials (Kelly and Russell, 2007). Subsequently, recombinant DNA technology was discovered and it became possible to enhance the safety and the efficacy through manipulation of the viral features to make them more tumor-specific. The first virotherapy study with genetically engineered virus was conducted in 1991 with herpes simplex virus type I (HSV-1) against experimental glioma model (Martuza et al., 1991) and a few years later the first clinical trial with engineered oncolytic adenovirus Onyx-015 took place (Ganly et al., 2000). During the last decade, the number of cancer therapy studies involving replication-selective oncolytic viruses has increased and currently there are many ongoing studies with more than 15 different species of viruses. Importantly, the first marketing approval for genetically modified oncolytic adenovirus H101 took place in China in November 2005 (Garber, 2006; Guo and Xin, 2006; Yin, 2006).

The ideal replication-selective oncolytic virus should infect and replicate only in tumor cells without harming the healthy neighboring cells. Also, its parental virus should be, at most, a mild and well characterized pathogen that should not integrate into the genome of the host cell. From the safety and uniform manufacturing point of view, a genetically stable virus would be desirable (Kirn et al., 2001). The ideal oncolytic agent could be administered in a remote site from the tumors and could be thus used as an anticancer agent against the metastatic forms of the disease (Parato et al., 2005).

The oncolytic viruses can be divided in two main groups, based on how the tumor specificity is gained. There are engineered tumor-selective viruses and naturally tumor specific viruses. The next sections describe the essential issues in virotherapy such as how the viruses elicit their tumor-specificity and also evaluate the safety and efficacy questions with different types of viruses.

2.2.2.1 GENETICALLY MODIFIED ONCOTROPIC VIRUSES

Viruses can be manipulated so that they selectively infect cancer cells without harming neighboring healthy cells and their virulence can also be attenuated by deleting parts of the viral genome. The changes in cancer cell features are used as targets when composing new viral agents to induce tumor-specific replication (Bell et al., 2002; Chiocca, 2002; Parato et al., 2005).
HSV-1 is one of the most widely studied oncolytic agents. It is an enveloped dsDNA virus with a large genome size (152kb) (Roizman and Sears, 1996). The wild type HSV-1 is known to be able to grow in neuronal tissues and to cause a number of diseases. These illnesses are rarely severe medical threats for healthy adults, but in the worse case scenario, HSV-1 can cause encephalitis. Natural infections either follow the lytic cycle or establish latency in neurons (Sundaresan et al., 2000). The advantages of HSV-1 are its large cloning capacity, neurotropism that allows efficient delivery to the CNS, the available anti-herpetic drug (acyclovir) in clinical use provides higher safety for the vector and most importantly, herpes virus genome does not integrate in the human genome (Aghi and Martuza, 2005; Markert et al., 2000). A variety of mutations has been introduced to reduce the neurotoxicity of HSV-1 and these mutated viruses represent potential candidates for replication and killing especially malignancies of CNS origin. The first engineered oncolytic vector dlspkt was based on HSV-1 and it was tested in an experimental glioma model. The mutant virus used in that study was lacking the TK-gene and in this way it exhibited attenuated neurovirulence. The tumor-targeting property of TK-deficient mutants was demonstrated by its severely impaired ability to replicate in non-dividing cells (normal non-dividing neurons and glia cells) (Martuza et al., 1991). Nevertheless, this first mutant displayed some weaknesses (some degree of neurotoxicity in normal neurons and insensitivity to anti-herpetic drugs) and therefore scientists have sought to develop safer and less neurovirulent mutants (Boviatsis et al., 1994). The γ34.5 mutant viruses have specific deletions in this particular neurovirulence gene and this alteration makes the virus highly attenuated and further targeted to replicate in cancer cells (Andreansky et al., 1998; Chou et al., 1990). The function of the γ34.5 gene product is to shut off the host cell’s protein synthesis by dephosphorylating the α-subunit of eukaryotic polypeptide chain initiation factor 2 (eIF2-α) in normal cells and thus it is needed for replication in non-dividing, quiescent cells (He et al., 1997). If this gene is lacking, the mutant virus is able to replicate only in dividing cells that have a defective PKR response, as is usually the case in cancer cells. Another favorable feature of these mutants is their sensitivity to anti-herpetic (GCV, acyclovir) drugs. Also, mutations in ICP6 gene (ribonuclotide reductase gene, RR which is needed for generation of deoxiribonucleotides in DNA synthesis) provides tumor specific features for HSV-1, since these viruses replicate only in rapidly dividing cells that provide the RR in complementation. These kinds of viruses cannot replicate in post-mitotic cells such as neurons (Hunter et al., 1999). Probably the safest HSV-1 mutant tested so far is the multi-attenuated G207 that has both γ34.5 and ICP6 genes mutated. The G207 mutant has been studied in epithelial ovarian cancer (Coukos et al., 2000), its safety has been demonstrated in rodents (Sundaresan et al., 2000) and primates (Hunter et al., 1999) and also in a clinical setting against glioblastoma multiforme.
(GBM) (Markert et al., 2000). Another promising mutant, HSV1716 (γ34.5 null mutant), has demonstrated safety and efficacy against human metastatic brain tumors (Detta et al., 2003) and high grade glioma (Harrow et al., 2004) and is currently being studied in a phase III randomized efficacy trial against recurrent GBM (Journal of Gene Medicine, http://www.wiley.co.uk/genmed/clinical/).

Adenoviruses are non-enveloped dsDNA viruses with genome size of approximately 38 kb. The adenovirus family consists of more than 50 serotypes that can cause only mild symptoms for humans. The vectors that are used in gene therapy are mostly based on the serotype 5 though some studies have been conducted with serotypes Ad2, Ad7, Ad4 as well as non-human viruses (Verma and Weitzman, 2005). The adenoviral replication cycle comprises of early and late phases, separated by the initiation of DNA replication. The early genes’ special role is to stimulate the infected cell to enter the cell cycle and to progress to the S-phase, in which the virus can utilize the host's DNA-replication machinery in order to replicate (Flint and Shenk, 1997). Two adenoviral early genes, E1A and E1B are essential for replication in normal cells and deletions in these genes have been shown to evoke tumor-specific replication (Everts and van der Poel, 2005; Kirn, 2000).

Two main approaches have been used for engineering conditionally replicative adenoviruses (CRAds) (Heise and Kirn, 2000). The first is to optimize the tumor-selectivity by deleting genes that are crucial for efficient viral replication in normal cells but not in malignant cells. The product of adenoviral early gene E1A competes with transcription factor E2F for binding to pRB, resulting in the release of E2F and the activation of the cell cycle via the transition from the G1 into the S-phase (Berk, 2005) (more detailed in Figure 3). One very common feature of cancer cells is the defective pRB route (Hanahan and Weinberg, 2000; Sherr, 1996). Thus, if an adenovirus has deletions in its E1A conserved region 2 (CR2), it can no longer replicate in cells with a normally functioning pRB pathway, but in cancer cells with defective pRB route it can replicate efficiently. Many of E1A adenoviral mutants have shown encouraging selective replication in different cancer types in vitro and in vivo (Fueyo et al., 2000; Heise et al., 2000). Also several derivates from the E1A mutants intended to further enhance the cancer cell killing have been generated by inserting either p53 (van Beusechem et al., 2002), GM-CSF (Bristol et al., 2003) or HSV-TK gene (Hakkarainen et al., 2006) as transgenes.

An alternative to achieve tumor-selective replication is through deletion of another early gene, E1B55kd. The adenoviral E1B55kd protein product is responsible for binding to the p53 tumor suppressor protein allowing the infected cell to bypass the cell cycle checkpoints. The natural
function of p53 pathway is to induce apoptosis in response to stress (such as viral infection) and DNA damage (Lane, 2001; Lane, 1992; Levine et al., 1991; Olson and Levine, 1994). The E1B55kd deleted adenovirus, known better by the names dl1520 and Onyx-015, should consequently be only able to replicate in cancer cells with mutated p53 (occurs in 60% of human cancers) (Biederer et al., 2002). It was proven earlier that there was a clear correlation between the p53 status and Onyx-015 replication (Bischoff et al., 1996) and the replication in primary cells was attenuated (Heise et al., 1997). However, it was later postulated that the p53 defect was not an absolute requirement for the function of Onyx-015, since it was replicating also in p53 positive cancer cells (Goodrum and Ornelles, 1998; Harada and Berk, 1999; Rothmann et al., 1998; You et al., 2000). The way that tumor cells support Onyx-015 replication is still under investigation and there are already several theories implicating mutations in genes (like \( p14^{ARF} \) and Mdm2) that can further regulate the p53 effects (Ries et al., 2000). Nonetheless, the potency of Onyx-015 has been shown in several tumor models (Bischoff et al., 1996; Rogulski et al., 2000). In addition, safety and efficacy has been studied in phase I/II clinical trials with i.t. administration (Ganly et al., 2000; Nemunaitis et al., 2001b) and intra-arterial injections (Reid et al., 2001). In China, very similar oncolytic agent to the Onyx-015 called H101 (the only difference is the slightly larger deletion in immune modulator E3 gene) was approved for marketing in November 2005 (Garber, 2006).

Another approach to evoke tumor-specific replication with adenoviruses is to use tissue/tumor-specific -promoters such as PSA promoter (Rodriguez et al., 1997), surviving promoter (Li et al., 2006) and human telomerase reverse transcriptase (hTERT) promoter (Kawashima et al., 2004) to drive the expression of the E1A. Based on this approach, a mutant adenovirus CV706 with PSA-selective replication against prostate cancer is now in a phase I clinical trial (DeWeese et al., 2001).

Adenoviruses are considered ideal oncolytic agents, especially from the viewpoint of safety (low pathogenicity, non-integrating) and manufacturing (high titer production and purification under GMP) (Heise and Kirn, 2000). On the other hand, the liver toxicity of the virus is a somewhat open question, but studies done with replication competent viruses have shown low toxicity and safety.
Figure 3. Cell cycle control mechanisms that are usually impaired in cancer cells are utilized in designing CRAds to obtain cancer specific replication. In normal cells the pRB regulates the G1 to S-phase checkpoint. When pRB is dephosphorylated, it binds to E2F (induces expression of genes needed for DNA synthesis) and in this way prevents the cell cycle progression. Adenoviral E1A competes with E2F for binding to pRB, releasing this way E2F into an active state and allowing the virus to replicate. When the viral E1A gene is mutated (like in AdΔ24), the virus can only replicate in cells that have defects in the pRB route. Also, adenoviral E1B-55kd interferes with the cell’s defense mechanism in a similar manner: it binds to p53 and inactivates it, preventing the normal function of p53 (apoptosis inducer → cell cycle arrest). Also in this case the E1B55kd deleted viruses (e.g. Onyx-015) can efficiently replicate in p53 defective cells, but not in normal cells (based on Everts and van der Poel, 2005).

2.2.2.2 NATURALLY OCCURRING ONCOTROPIC VIRUSES

Cancer cells and their specific features make them optimal hosts in which viruses can replicate, since in these cells the antiviral responses (i.e. inhibition of apoptosis, activated Ras pathway and defective IFN pathway) are often impaired. The role of activated Ras pathway and defective IFN pathway has been shown to play a major role in the replication competence of naturally tumor-selective viruses (most of them are RNA viruses) (Figure 4.). These wild type viruses (reovirus, NDV and measles virus) have been used as anticancer agents in recent virotherapy studies (Chiocca, 2002; Everts and van der Poel, 2005; Russell, 2002).
Figure 4. The essential role of IFN- and PKR-pathways in viral infection and virotherapy approaches. The double stranded RNA dependent protein kinase (PKR) is an important downstream effector in the interferon (IFN) signaling pathway, which is part of host cells’ defense system against viral stimuli. PKR can bind to viral dsRNA and this leads to autophosphorylation of the PKR-homodimer and the activated PKR phosphorylates eIF-2α, which leads to inhibition of protein synthesis. However, this mechanism is usually impaired in cancer cells and it is through this pathway that some oncolytic viruses gain their tumor specificity: reovirus is dependent on the activated Ras pathway, vesicular stomatitis virus (VSV) is dependent on the inactivated IFN pathway. Furthermore, some viruses have evolved mechanisms to circumvent antiviral responses e.g. like adenoviral VA-RNA and herpes viral γ34.5 (modified from Chiocca, 2002 and Everts and van der Poel, 2005).

The human Reovirus is a double-stranded RNA virus that causes only mild (usually asymptomatic) upper respiratory and gastrointestinal infections (Nilbert et al., 1996). This virus preferentially replicates in many transformed cells. The mechanism under the tumor-specific replication was shown to be sensitive to overexpression of EGF-receptor (EGFR) or $\text{verbB}$-oncogene which are upstream of the Ras-signaling cascade (Figure 4.) (Strong and Lee, 1996; Strong et al., 1993). Reoviruses’ opportunistic utilization of this activated signal transduction pathway was also confirmed in a study where reovirus resistant cells became permissive to reovirus infection after being transformed with the $\text{Ras}$-oncogene. The role of PKR-inactivation
in activated Ras signaling pathway was also shown to be essential, since the inhibition of the antiviral activity of the cells enhanced reovirus susceptibility (Strong et al., 1998). Reovirus has been shown to infect several established human cancer cells \textit{in vitro} and \textit{in vivo} (Hirasawa et al., 2002; Norman et al., 2002; Wilcox et al., 2001). Direct and remote injections of reovirus have been proved to eradicate subcutaneous tumors from SCID-mouse (U87, v-erbB-transformed cells) and from immunocompetent mouse (Ras-transformed C3H-10T1/2 fibroblasts). It was shown that reovirus did not induce antitumor immune responses and thus the immune system was not positively involved in tumor eradication (Coffey et al., 1998). Furthermore, the immune suppression with cyclosporine A led to enhanced reovirus replication and tumor regression in immunocompetent animals (Hirasawa et al., 2003; Smakman et al., 2006).

The encouraging results from pre-clinical studies, the well-known mechanism behind the selective replication in tumor cells (activated proto-oncogene \textit{Ras} occurring in 30\% of all human tumors [Bos, 1989]) and the relatively low pathogenic nature makes reovirus a potential candidate for cancer virotherapy. Currently, reovirus is being studied in phase I clinical trials with i.v. administration and in phase II trials against recurrent malignant glioma (Carlson et al., 2005; Shmulevitz et al., 2005).

Another promising naturally tumor-specific virus, Newcastle disease virus (NDV), belongs to the enveloped chicken paramyxoviruses. It is a single-stranded RNA virus with negative polarity and it is mild pathogen for humans (Sinkovics and Horvath, 2000). This oncolytic virus also exploits the cancer cells’ activated Ras pathway and defective IFN pathway (Lorence et al., 1994a). The strain 73-T (passaged in mouse ascites cells for 73 cycles) was shown to eradicate several tumor types in different cancer cell cultures and immunocompromised animal tumor models (Lorence et al., 1994a; Lorence et al., 1994b; Phuangsab et al., 2001). During the last few years, different strains of NDV (PV701, NDV-HUJ) have entered into clinical phase I/II studies with some degrees of success (Freeman et al., 2006). The advantages of the PV701 strain are its ability to induce T-cell mediated specific antitumor immunity and the activation of innate immune system such as tumoricidal macrophages to participate in tumor cell destruction (Termeer et al., 2000). NDV has been used also as a viral oncolysate (suspension of virus and tumor cells) to trigger immune response against the tumor (Sinkovics and Horvath, 2000) and this approach is now being evaluated in clinical studies (Batliwalla et al., 1998). The advantages of NDV virus as an anticancer agent are the rapid oncolysis, low pathogenicity for humans and, most importantly, its ability to trigger antitumor immunity even when administered systemically.
Enveloped paramyxovirus, measles virus (MV) has been shown to have oncolytic activity in cancer cells. Wild type MV causes a well known disease that consists of fever, rash and transient immune suppression. The wild type virus uses signaling lymphocyte activation molecule (SLAM) receptors to infect and replicate in the activated T- and B-cells (Tatsuo et al., 2000). Apparently therefore there have been case reports about regression of malignant lymphomas after natural measles infection (Bluming and Ziegler, 1971; Taqi et al., 1981) reviewed by (Aghi and Martuza, 2005). The attenuated strain of MV (Edmonston strain, the vaccine strain) infects also by targeting the CD46 receptors (Dorig et al., 1993) that are to some extent overexpressed in human tumors (especially ovarian tumor cells) (Peng et al., 2002). The derivates of this vaccine strain can be considered as being relatively safe since the live attenuated MV vaccines have been used for 30 years for humans. Since most adults are immune to MV, Gröte and coworkers have proved (Gröte et al., 2001) that even in the presence of anti-MV antibodies, the tumor eradication can occur efficiently. The recombinant MV expressing carcinoembryonic antigen (CEA, used as a marker) showed enhancement of survival of mice bearing intracranial U-87 glioma (Phuong et al., 2003). This virus has entered into clinical phase I studies against ovarian cancer and GBM (Journal of Gene Medicine, http://www.wiley.co.uk/genmed/clinical/).

Yet another promising new candidate for a virotherapeutic agent could be the enveloped alphavirus SFV that has a single-stranded RNA genome with positive polarity. The genome contains two open reading frames (ORF): the first one encodes the non-structural proteins (nsp1-nsp4) that are responsible for transcription and replication of viral RNA. The second ORF codes for the structural proteins (E1, E2, E3 and 6k) that are required for encapsidation of the genome and proper assembly into enveloped particles (Strauss and Strauss, 1994). The latter ones are not necessary for viral genome replication but are needed for virus propagation. In replication deficient SFV systems (replicons), the viral structural proteins are replaced with a transgene and these vectors are able to carry out one round of replication (Lundström, 2005; Rheme et al., 2005; Wahlfors et al., 2000). The SFV replicons have mostly been utilized in recombinant protein production due to their efficient and rapid replication cycle in the cytoplasm, but it has been also studied in different cancer gene therapy applications: as a self replicating RNA vaccine (Ying et al., 1999), as apoptosis inducing replicons with or without apoptosis inducer Bax-gene (Murphy et al., 2000; Murphy et al., 2001) and in an immune stimulatory manner expressing IL-12 or p62-6k (SFV structural protein) (Chikkanna-Gowda et al., 2005; Ren et al., 2003; Smyth et al., 2005). SFV does possess the desired features for an oncolytic agent: rapid replication cycle (lysis within 8h), mild pathogenicity for humans and a broad host range. In particular, the VA7-EGFP (Vähä-Koskela et al., 2003) vector that is based on the avirulent strain of SFV A7 (74) has
been shown to eradicate subcutaneous melanoma tumors from mice irrespective of the administration route (Vähä-Koskela et al., 2006). The mechanism to account for the tumor targeting properties of SFV is not yet fully defined, but another alphavirus commonly used as a vector, Sindbis virus, has been shown to target tumor cells through the laminin receptors on the cell surface (Tseng et al., 2004). Furthermore, like most other RNA viruses, alphaviruses are known to be sensitive to the type I IFN response, suggesting that defective IFN pathway occurring in many cancer cells provides a permissive environment to favour efficient replication (Nilsen et al., 1980).

Virotherapy is a growing field and Table 1 describes some other oncolytic viruses that are being evaluated for their anticancer properties.

Table 1. Other oncolytic viruses

<table>
<thead>
<tr>
<th>VIRUSES</th>
<th>GENOME</th>
<th>MECHANISM BEHIND TUMOR SPECIFICITY</th>
<th>REFERENCE</th>
</tr>
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<tbody>
<tr>
<td>Myxoma virus</td>
<td>dsDNA</td>
<td>Not defined, probably defective IFN pathway</td>
<td>(Lun et al., 2005)</td>
</tr>
<tr>
<td>Mumps virus</td>
<td>ssRNA</td>
<td>Not defined, receptors that mumps virus uses are highly expressed in malignant cells, antitumor immunity?</td>
<td>(Myers et al., 2005; Yan et al., 2005)</td>
</tr>
<tr>
<td>Autonomous Parvovirus</td>
<td>ssDNA</td>
<td>IFN-resistant tumors</td>
<td>(Olijslagers et al., 2001)</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>dsDNA</td>
<td>TK-gene deletion</td>
<td>(Hung et al., 2007)</td>
</tr>
<tr>
<td>Sindbis virus</td>
<td>ssRNA</td>
<td>Laminin receptors, defective IFN pathway, activated Ras pathway?</td>
<td>(Unno et al., 2005)</td>
</tr>
<tr>
<td>VSV</td>
<td>ssRNA</td>
<td>Loss of cell cycle control, defective IFN pathway</td>
<td>(Power et al., 2007; Stojdl et al., 2000)</td>
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<tr>
<td>Influenza virus</td>
<td>ssRNA</td>
<td>NS1 deleted virus, replicates in cells with defective IFN pathway</td>
<td>(Muster et al., 2004)</td>
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2.3 CHALLENGES IN VIROTHERAPY

2.3.1 EFFICACY

The low efficacy in conventional gene therapy has inspired many workers to use replication competent vectors (Kirn et al., 2001). One of the principal aims of using selectively replicative viruses as anticancer agents was to gain access to the malignant tissues and metastases by injecting the virus to a remote site. Many pre-clinical studies have demonstrated the potential
efficacy in different animal tumor models (Bischoff et al., 1996; Bristol et al., 2003; Hirasawa et al., 2002) and safety and selective replication has been shown with both types (naturally tumor-specific viruses and genetically engineered viruses) in clinical trials. Nevertheless, the clinical phase I/II trials have shown only modest antitumoral activity, even in i.t. treated cancers with replication competent viruses (Onyx-015 and G207) (Ganly et al., 2000; Markert et al., 2000). Therefore, the effective treatment to cure metastatic cancer is still not a reality in most cases and further research is needed to make virotherapy work also systemically (Liu and Kirn, 2007; Power et al., 2007). Even though the initial concern was safety, it has transpired that the real major problem is the need to achieve better efficacy (Aghi and Martuza, 2005). Before one can improve the efficacy it is mandatory to understand better the dynamics between virus replication, immune mediated viral clearance and the three dimensional nature of tumor growth (Wein et al., 2003).

The greatest challenge in virotherapy is the immune system (this will be discussed later in a separate paragraph). In addition, other factors such as physical barriers; necrotic areas, thickness of the capsule surrounding the nodules, and the extracellular matrix may limit virus spread within the tumor mass (Ries and Brandts, 2004). The size of the tumor nodules also correlates with the therapy outcome, since oncolysis is known to be a dynamic process where the replication kinetics of the virus and the growth rate of the tumor cells affect each other (Gröte et al., 2001). A mathematical model has been developed to simulate the race between oncolysis and the spread of the virus versus tumor cell proliferation. The model demonstrates that the infection has to diffuse throughout the tumor in order to control its growth (Wein et al., 2003). If the viral particles are too large, if there is low expression of virus receptors on the surface of the cancer cells and if there is a heterogenic population of cells in the tumors (almost 50% of the tumor mass can be normal cells with normal antiviral actions) are all factors that might explain partly their ineffective spread within the tumor (Davis and Fang, 2005).

One factor that reduces the general utility of CRAds is insufficient expression of adenoviral receptors (CAR) on the surface of cancer cells (Kim et al., 2002; Qin et al., 2003). Thus, to achieve tumor-selective binding, researchers have developed CRAds that target the cancer cells via other receptors such as CD46 (Suominen et al., 2006) or integrins with the aid of an inserted integrin binding RGD-motif (Kanerva et al., 2002; Sarkioja et al., 2006; Witlox et al., 2004). Although oncolytic vectors do not require any inserted transgenes to replicate and kill their target cells, their anticancer effect can be further enhanced by inserting genes such as immune
response-modifying genes (Kim et al., 2006) or tumor suppressor genes (van Beusechem et al., 2002).

The concept of using a single agent therapy has been noted as being unsuccessful in achieving a complete cure and thus combination therapy with the existing conventional modalities probably offers the greatest potential. Combining virotherapy agent e.g. Onyx-015 with a chemotherapy agent such as cisplatin or 5-FU, enhanced the survival of animals with experimental cancer models compared to either of the treatments alone (Heise et al., 1997). Synergistic effects between replication competent herpes virus and radiotherapy also enhanced the antitumoral effects in experimental NSCLC (Adusumilli et al., 2005). Enhanced responses have also been reported in clinical studies with Onyx-015 combined with 5-FU in the treatment of patients with metastatic colorectal carcinoma (Reid et al., 2001).

Finally, it has to be mentioned that cancer patients that are enrolled in clinical studies are mostly those in whom conventional treatment has failed and they very often carry highly metastatic stage III-IV cancers. Therefore, it is very difficult to obtain a complete cure or even a good response in these patients. Since it is well known that oncolysis is a dynamic process where the tumor growth competes with viral replication, it appear to be crucial to test the clinical utility of these treatment strategies in patients with less advanced cancers. In fact, this was proposed already more than 30 years ago, when Asada referred to a successful therapy with mumps virus in 1974 and speculated on, what the outcome would have been with earlier stage cancer patients (Asada, 1974).

2.3.1.1 IMMUNOLOGICAL ASPECTS

Immune responses represent one of the major challenges in virotherapy. Many oncolytic viruses do exhibit clear efficacy when administered directly into tumors, but when the treatment is performed systemically (e.g. intravenously), the virus is very vulnerable to the components that are circulating in the bloodstream, such as complement proteins (Wakimoto et al., 2002), phagocytic cells and neutralizing antibodies (Fulci et al., 2006; Ikeda et al., 1999). Moreover, in the case of adenoviruses, the excessive uptake of the virus by the liver after systemic delivery inhibits the response and triggers toxic side effects (Brunetti-Pierri et al., 2004).

Even though promising results have been obtained from animal studies, previous reports from clinical studies indicate only a few examples where positive responses have been obtained with systemically administered oncolytic agents. Most of these viruses belong to the group of naturally oncotropic viruses discussed earlier (Table 2.).
Table 2. Systemic efficacy with oncolytic viruses as a single agent (based on Liu and Kirn, 2007).

<table>
<thead>
<tr>
<th>VIRUS SPECIES</th>
<th>AGENT</th>
<th>DESCRIPTION (deletions in viral backbone /therapeutic transgene)</th>
<th>SYSTEMIC TUMOR RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Onyx-015</td>
<td>E1B-55kd- and E3B- / no transgene</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>H 101</td>
<td>E1B-55kd- and E3- / no transgene</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>CV706</td>
<td>PSE-E1A- and E3 / no transgene</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>AdΔ24</td>
<td>E1A-CR2- and E3- / no transgene</td>
<td>No</td>
</tr>
<tr>
<td>HSV</td>
<td>I716</td>
<td>ICP34.5/ no transgene</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>G207</td>
<td>ICP34.5; ICP6 / no transgene</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>OncoVex</td>
<td>ICP34.5; ICP6 / GM-CSF</td>
<td>No (i.v. → pending)</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>Vaccine strain</td>
<td>No / no transgene</td>
<td>Yes (i.t., i.v. or vaccination)</td>
</tr>
<tr>
<td></td>
<td>JX-594</td>
<td>Tk / GM-CSF</td>
<td>Yes (i.t.) (i.v.→ pending)</td>
</tr>
<tr>
<td>Measles</td>
<td>Vaccine strain</td>
<td>No / no transgene</td>
<td>Yes (Vaccination)</td>
</tr>
<tr>
<td></td>
<td>MV-CEA</td>
<td>No / CEA (for monitoring)</td>
<td>No (i.v. and i.p. → pending)</td>
</tr>
<tr>
<td>Mumps</td>
<td>Vaccine strain</td>
<td>No / no transgene</td>
<td>Yes (i.v.)</td>
</tr>
<tr>
<td>West Nile</td>
<td>Vaccine strain</td>
<td>No / no transgene</td>
<td>Yes (i.m.)</td>
</tr>
<tr>
<td>NDV</td>
<td>PV701</td>
<td>No / no transgene</td>
<td>Yes (i.v) (i.t.→ pending)</td>
</tr>
<tr>
<td></td>
<td>HUJ</td>
<td>No / no transgene</td>
<td>Yes (i.t.)</td>
</tr>
<tr>
<td>Reovirus</td>
<td>Reolysin</td>
<td>No / no transgene</td>
<td>Yes (i.v.)</td>
</tr>
</tbody>
</table>

2.3.1.1.1 Immune system suppression

There are many studies focused on ways to achieve enhanced oncolytic efficacy by suppressing the unwanted effects of the components that inhibit the powerful replication (Figure 5.). Complement can be transiently neutralized by administration of cobra venom factor (CVF) (Ikeda et al., 2000), and cyclophosphamide (CPA) (Fulci et al., 2006; Ikeda et al., 2000) has been shown to inhibit the intratumoral infiltration of mononuclear cells and levels of IFNγ. One important function of CPA is the reduction of the dose of the oncolytic agent needed for efficient replication (Kambara et al., 2005). The increase in the efficacy attained with CPA means that the safety is also enhanced, because the viral dose can be reduced. Other immune suppressors, such as cyclosporine (Hirasawa et al., 2003; Smakman et al., 2006) have been used for eliciting more powerful replication within tumor by those viruses that are weakened by the immune system (reovirus, VSV, SFV).
Another attractive alternative to circumvent the immune system is to use cell-based delivery of the virus. This means that the individual’s own cells carry the viral vector to the tumor site (Cole et al., 2005; Garcia-Castro et al., 2005). This type of immune evasion has been used with oncolytic VSV therapy, where carrier cells (also called Trojan horses) deliver the virus to lung metastases and in this way, the virus bypasses the obstacles between the tumor and the injection needle. If there is a need to deliver the virus also to different sites (for example, metastases) through the microcapillaries, the carrier cells need to be small enough, perhaps representing blood cell types (Power et al., 2007). Another immune evasion strategy which has been used in a phase I/II glioma study involves encapsulation of SFV replicons into non-immunogenic liposomes (Ren et al., 2003). The removal of adenoviral E3 region from most adenovectors, has been claimed as being a major error (Davis and Fang, 2005), since the E3 is the natural immune modulator protein of the virus (Dimitrov et al., 1997). The presence of E3 could limit antigen presentation, interfering with MHC-I and in this way prevent the inflammation and CTL detection and this could lead to enhanced efficacy (Benedict et al., 2001; Davis and Fang, 2005).
2.3.1.1.2 Immune system as a key player

However, the immune response against virus infected tumor cells can also be therapeutically beneficial. Certain viruses, like NDV, appear to elicit antitumor immune responses and this reaction is actually capable of inducing systemic antitumor effects. The NDV oncolysates discussed earlier are good examples of ways to trigger a systemic antitumor response against the tumor-antigens (Savage et al., 1986). Also, in case of HSV-1 (G207), it has been shown that to obtain better response in immunocompetent animals, local cytotoxic replication is needed. Furthermore, a systemic anti-tumor immune response (CTL-activity) that produces an antitumor vaccination-type of effect (Toda et al., 1999; Todo et al., 1999) is likely to be important. The use of replicative viruses in co-operation with the immune system in destroying tumors has been shown to represent a promising approach. For example, GM-CSF armed attenuated (TK-) vaccinia virus injected i.v. has been noted to induce a tumor specific CTL response against immunocompetent rat and rabbit liver tumors with encouraging results (Kim et al., 2006). In the case of vaccinia, the response is triggered even after intravenous administration, since the virus disseminates through blood and has evolved into an extracellular, enveloped form that is resistant to antibodies and complement mediated neutralization (Vanderplasschen et al., 1998). In the future it is likely that the viruses that are able to disseminate through blood will be the ones that will achieve better systemic responses (Liu and Kirn, 2007). A study done with intratumorally administered targeted adenoviral vector expressing GM-CSF was also shown to induce potent antitumoral immune responses, resulting in tumor eradication (Bristol et al., 2003).

2.3.2 SAFETY

A variety of different replication competent viruses have been evaluated for their potential as anticancer agents, each of them with distinct types of replication mechanisms but all aiming at the same target: destroying cancer cells rapidly, thoroughly and even from remote site. Several viruses have shown favorable safety profiles already in clinical trials (Markert et al., 2000; Nemunaitis et al., 2001a; Pecora et al., 2002; Reid et al., 2001). Of more than 300 patients treated in phase I/II clinical trials, only one treatment-related death has been reported to date from intravenous NDV treatment (and even then the death may have resulted from too rapid tumor lysis) (Pecora et al., 2002). In addition, the side effects after the treatments have mostly been flu-like symptoms (fever, headache) (Freeman et al., 2006; Ganly et al., 2000; Pecora et al., 2002) and they can be considered as mild if they are compared to the distressing side effects evoked by conventional cancer therapies. Anyhow, every case with a novel anti-tumor agent is a
whole new case and the safety issues have to be unquestionably carefully evaluated to prevent
the risks.

As mentioned earlier, desirable safety features for oncolytic viruses would be a non-integrating
nature with a stable genome and mild pathogenicity for humans. A well-known and well-
characterized nature of the wild type virus and its pathogenicity are also important features.
Furthermore, if one resorts to more pathogenic viruses like HSV-1, then the deletions in the
virulent genes (both γ34.5 genes) are essential. Single gene mutant HSV-1 viruses still maintain
the risks of wild type reversion (Bennett et al., 2002). Even when working with multi-attenuated
strains like G207, it is necessary to verify that the patient's latent viruses do not become
reactivated and cause a clinical disease after inoculation with the mutant G207 virus (Hunter et
al., 1999; Sundaresan et al., 2000), especially since the HSV-1 is ubiquitous in people over 30
years of age (90 % have detectable HSV-1 antibodies) (Andreansky et al., 1996).

Some of the oncolytic viruses are animal viruses that nonetheless kill efficiently human tumor
cells but do not cause any known disease in humans. There is a highly theoretical risk that these
viruses could evolve into new human viruses and that could cause a disease and spread into non-
treated individuals (Kelly and Russell, 2007). This possibility is difficult to evaluate but at least
the widely studied NDV (that is a zoonotic avian virus) has been shown to be safe in many
clinical studies (Batlivala et al., 1998; Freeman et al., 2006). In that respect, the use of
genetically stable viruses should be preferred. RNA viruses are known to be more unstable and
are sensitive to mutations and intermolecular recombination. One example of the mutational risk
becoming a reality comes from the live attenuated strain of poliovirus that has been successfully
and widely used for vaccination in the world. However, its inherited sensitivity to point
mutations has been revealed to be linked to rare cases of vaccination-related paralytic
poliomyelitis (Cherkasova et al., 2003). Therefore, when working with viruses, the benefits and
risks have to be evaluated thoroughly.

One very important issue in virus safety is the ability to produce high-titer virus preparations
according to Good Manufacturing Practice (GMP) guidelines for clinical studies (Kirn, 2000).
When proceeding to clinical studies, the virus stocks should be easy to purify and concentrate,
since large quantities will be needed for human use. The quality, safety and stability of the
production have to be on as solid a basis as possible, especially when the “medicine” is a live
virus with a mutational potential (Working et al., 2005). This, again, raises the important issue of
genome stability which was discussed above. The manufacturing of clinically suitable
vector/virus in virotherapy is a challenge and therefore the use of commercial vaccination strains
as anticancer agents has been proposed by Myers and co-workers (Myers et al., 2005) since these strains have already been approved for human use and thus possess extensive safety records.

## 2.3.2.1 Improving Safety and Control

There are several alternative options available to further improve safety. Most vectors that are used in virotherapy are conditionally replicating, meaning that they are already tumor specific, but further enhancement of the specificity is always possible and desirable. One approach is to target the vector transcriptionally (Ko et al., 2005) by using a tissue/tumor specific promoter such as PSA (Rodriguez et al., 1997), survivin (Stoff-Khalili et al., 2005), E2F-1 (Jakubczak et al., 2003) or by using promoters such as hexokinase II (HKII) (Katabi et al., 1999) that respond to the different microenvironment and physiology of the cancer cells (such as glucose catabolism). Another option is to target the cancer cell transductionally by modifying the virus to recognize specific cell surface molecules e.g. via receptors that are expressed by cancer cells such as CD40, highly expressed in ovarian cancer cells (Hakkainen et al., 2003) or specific for alpha-folate receptor (overexpressed in nonmucinous ovarian cancer) (Hasegawa et al., 2006). Furthermore, combinations of these different targeting approaches should result in optimized cancer specific gene therapy agents with increased safety features (Rots et al., 2003). However, occasionally increasing the safety by inserting new genetic material into the virus/vector and deleting parts of the virus, can lead to decreased efficacy, since the biology of the life cycle can be adversely affected (Vile et al., 2002).

After injection of a replication competent vector to the patient, further control over the virus and its replication is not possible from the outside and this can be viewed as a risk. There are a few alternatives on how to minimize this type of risk. One option is to incorporate specific suicide genes (HSV-TK/GCV) to provide the possibility to eliminate the virus should something go awry (Wildner and Morris, 2000). Another possibility to control the oncolysis from the outside is to use a tetracycline-controlled transactivator-transrepressor system. In this system, the replication of the adenovirus is switched on by administration of doxycycline and switched off by withdrawal of doxycycline (Fechner et al., 2003). Especially from the patients’ point of view, it is desirable to have a safety switch to stop the replication of the virus and to eliminate it from the body.
2.3.3 ANIMAL MODELS

The animal models used in testing the efficacy and safety of new anticancer agents have a great influence on the outcome of the study. Cancer therapy studies are carried out in two types of tumor models; 1. human tumor cells growing in an immunosuppressed animal or 2. syngenic tumor cells growing in an immunocompetent animal. Neither of these models are identical to actual human tumors and for example factors such as complement activation against oncolytic agents have been shown to vary extensively between different species (Wakimoto et al., 2002). Both models have their strengths and weaknesses. Studies with tumor models in severely combined immunodeficient (SCID) mice provide the possibility to analyze tumor-virus interactions without the interference of the immune system (Vähä-Koskela et al., 2006). Due to the lack of a fully functional immune system, the results obtained are poor indicators of what to be expected in an immunocompetent human being, even though many cancer patients with advanced disease are more or less immunocompromised (Zhang et al., 2005). Other immunodeficient animals used widely in cancer therapy studies are so-called nude mice, which lack the functional T-cell response and therefore cannot reject human cell xenografts. Many positive responses gained from nude mouse tumor models have shown a lack of correlation with results gained from clinical studies (Kelland, 2004). In particular, subcutaneous tumors that do not usually metastasize are not relevant models for metastatic cancers. For this reason, orthotopic models are favored (Fukunaga et al., 2002; Lun et al., 2005).

One more issue regarding the relevance of a tumor model is the safety aspect. Though pre-clinical studies have demonstrated safety and efficacy in immunocompetent animals, it is still not comparable to the situation with cancer patients that are typically elderly, immunocompromised and highly vulnerable to infections (Davis and Fang, 2005). One further factor is that human viruses do not cause similar effects in animals as they evoke in humans and vice versa. Thus, when one has tested the safety of a human virus in an animal model and evaluated it to be free of problems, is it possible to conclude that the agent is also safe in humans? Or, from the other point of view, if we obtain alarming results from a safety study carried out in a rodent model with a rodent virus, can we conclude that it is unsafe and eliminate one potential anticancer agent?

The problem with few human viruses like adenovirus and MV is that there are no proper immunocompetent tumor models available for studying how a functional immune system would affect the therapy and whether tolerance will be developed to the oncolytic virus. Today most pre-clinical studies are conducted with nude mouse human xenografts, because rodent cells are
several orders of magnitude less susceptible to human adenovirus than human cells (Biederer et al., 2002; Fechner et al., 2003). One promising model developed for adenoviral studies is the Syrian hamster model that is both immunocompetent and replication-permissive to adenoviruses, therefore allowing further studies with syngenic tumor cells in animal with a functional immune system (Thomas et al., 2006). As long as there are no better models at hand, we have to continue using the best model available but to keep in mind that is unwise to draw too general conclusions about the safety and efficacy of the therapy (Vähä-Koskela et al., 2007).
3 AIMS OF THE STUDY

The general aim was to evaluate, whether gene therapy and virotherapy could be used as novel treatments against NSCLC. This study was focused on the fundamental issues of experimental cancer therapies: safety, efficacy and establishment of a better tumor model of NSCLC for pre-clinical studies.

The specific aims of this thesis were:

To evaluate the potency of adenoviral mediated HSV-TK/GCV suicide gene therapy against NSCLC in vitro and in vivo (I)

To target HSV-TK/GCV therapy transcriptionally to cancer cells with the human hexokinase II promoter, with a special emphasis on targeting human NSCLC cells (II)

To study the oncolytic potency of SFV virotherapy in NSCLC in vitro and in vivo, and to evaluate the impact of a fully functional immune system to SFV virotherapy in an immunocompetent glioma model (III)

To establish an orthotopic nude mouse model of human NSCLC and to study the response to SFV virotherapy in experimental lung cancer (IV)
4 MATERIALS AND METHODS

The materials and methods used in articles I-IV are briefly described below and are explained more thoroughly in the original publications.

4.1 CELLS

The cell lines used in studies (I-IV) are listed in Table 3. All cell lines were adherent and were cultured in +37°C under 5% CO₂. The recommended growth protocols are described in more detail in the cited articles.

Table 3. Cell lines used in studies I-IV. (Grouped into cancerous, normal and producer cell lines). The cells are of human origin, unless otherwise indicated.

<table>
<thead>
<tr>
<th>CANCER</th>
<th>ORIGIN</th>
<th>DESCRIPTION AND SOURCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>Lung</td>
<td>Adenocarcinoma (ATCC CCL-185)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>A172</td>
<td>Brain</td>
<td>Glioblastoma (ATCC CRL-1620)</td>
<td>II</td>
</tr>
<tr>
<td>BT4C</td>
<td>Brain</td>
<td>Rat glioma cells (Laerum et al., 1977)</td>
<td>III</td>
</tr>
<tr>
<td>Calu-3</td>
<td>Lung</td>
<td>Adenocarcinoma (ATCC HTB-55)</td>
<td>I</td>
</tr>
<tr>
<td>DU-145</td>
<td>Prostate</td>
<td>Carcinoma (ATCC HTB-81)</td>
<td>II</td>
</tr>
<tr>
<td>NCI-H1650</td>
<td>Lung</td>
<td>Adenocarcinoma (ATCC CRL-5883)</td>
<td>I</td>
</tr>
<tr>
<td>NCI-H23</td>
<td>Lung</td>
<td>Adenocarcinoma (ATCC CRL-5800)</td>
<td>I</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Lung</td>
<td>Large cell lung cancer (ATCC HTB-177)</td>
<td>I, II</td>
</tr>
<tr>
<td>NCI-H520</td>
<td>Lung</td>
<td>Squamous cell lung cancer (ATCC HTB-182)</td>
<td>I, II</td>
</tr>
<tr>
<td>NCI-H661</td>
<td>Lung</td>
<td>Large cell lung cancer (ATCC HTB-183)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>OV-4</td>
<td>Ovary</td>
<td>Adenocarcinoma (Gift from David T. Curiel)</td>
<td>II</td>
</tr>
<tr>
<td>PC3</td>
<td>Prostate</td>
<td>Adenocarcinoma (ATCC CRL-1435)</td>
<td>II</td>
</tr>
<tr>
<td>SKOV 3 ipl.</td>
<td>Ovary</td>
<td>Ovarian cancer (Gift from David T. Curiel)</td>
<td>II</td>
</tr>
<tr>
<td>SW900</td>
<td>Lung</td>
<td>Squamous cell lung cancer (HTB-59)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>TE671</td>
<td>Brain</td>
<td>Medulloblastoma (ATCC CCL-136)</td>
<td>II</td>
</tr>
</tbody>
</table>

NORMAL

| NHBE   | Lung   | Normal human bronchial epithelial cells (Clonetics) | II |
| NHFL   | Lung   | Normal human lung fibroblast (Clonetics) | II |

PRODUCER

| 293    | Kidney | Epithelial cells (ATCC CRL-1573) | II |
| 293T   | Kidney | Epithelial cells + SV40T antigen (ATCC CRL-11268) | I |
| BHK-21 | Kidney | Baby hamster kidney cells (CCL-10) | III, IV |
4.2 VIRAL VECTORS AND VIRUSES

The vectors used in the studies I-IV are listed in Table 4. The adenoviral vectors were AdTK-GFP (Loimas et al., 2000) and Ad5-Δ24TK-GFP (Hakkarainen et al., 2006) constructed and produced in our laboratory. The second-generation lentiviral vectors (kind gifts from Professor Didier Trono, University of Geneva, Switzerland) were used to create WOX-TK-GFP (Meriläinen et al., 2005) which was further used to clone the targeted vectors (hHKII1820, hHKII982 and hHKII352). The replication competent virus SFV A7(74) (Tuittila et al., 2000) and vector VA7-EGFP (Vähä-Koskela et al., 2003) were kind gifts from Docent Ari Hinkkanen (Åbo Akademi University, Turku, Finland). The detailed viral productions and titer determination can be found for adenovirus (I & IV), lentivirus (I & II), and SFV (III & IV).

Table 4. Viral vectors used in studies I-IV.

<table>
<thead>
<tr>
<th>ADENOVIRAL VECTORS</th>
<th>PROMOTER</th>
<th>DESCRIPTION</th>
<th>TRANSGENE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdTK-GFP</td>
<td>CMV</td>
<td>Serotype 5 based, first generation E1/E3 deleted adenoviral vector</td>
<td>TK-GFP</td>
<td>I</td>
</tr>
<tr>
<td>Ad5-Δ24TK-GFP</td>
<td>CMV</td>
<td>Conditionally replicative, serotype 5 based, E3 deleted adenoviral vector, contains 24bp deletion in E1A site</td>
<td>TK-GFP</td>
<td>IV</td>
</tr>
<tr>
<td>SFV VIRUSES AND VECTORS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VA7-EGFP</td>
<td>26S</td>
<td>Replication competent vector based on attenuated Semliki Forest virus strain A7(74)</td>
<td>EGFP</td>
<td>III, IV</td>
</tr>
<tr>
<td>SFV A7 (74)</td>
<td>26S</td>
<td>Replication competent virus based on attenuated Semliki Forest virus strain A7(74)</td>
<td></td>
<td>III</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LENTIVIRAL VECTORS</th>
<th>TRANSGENE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>hEF1α</td>
<td>TK-GFP</td>
<td>II</td>
</tr>
<tr>
<td>hHKII-1820 1820 bp fragment of hHKII</td>
<td>Second generation, VSV-G pseudotyped lentiviral vector, containing woodchuck hepatitis virus post-transcriptional element (WPRE)</td>
<td>II</td>
</tr>
<tr>
<td>hHKII-982 982 bp fragment of hHKII</td>
<td>Second generation, VSV-G pseudotyped lentiviral vector, containing WPRE</td>
<td>II</td>
</tr>
<tr>
<td>hHKII-352 352 bp fragment of hHKII</td>
<td>Second generation, VSV-G pseudotyped lentiviral vector, containing WPRE</td>
<td>II</td>
</tr>
</tbody>
</table>
4.3 IN VIVO EXPERIMENTS

All the performed animal studies were approved by the Animal Care and Use Committee of University of Kuopio.

Table 5. Animal models in studies I-IV. All animals were purchased from Harlan, Netherlands.

<table>
<thead>
<tr>
<th>TUMOR MODEL</th>
<th>CELLS</th>
<th>STRAIN</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous/Immunodeficient</td>
<td>A549 (3x10⁶)</td>
<td>NMRI-foxn-1 (Age&gt; 8weeks)</td>
<td>I, III</td>
</tr>
<tr>
<td></td>
<td>NCI-H661 (2 x 10⁶)</td>
<td>NMRI-foxn-1 (Age&gt; 8weeks)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>SW900 (5.5 x 10⁶)</td>
<td>NMRI-foxn-1 (Age&gt; 8weeks)</td>
<td>I</td>
</tr>
<tr>
<td>Orthotopic/Immunodeficient</td>
<td>A549 (1 x 10⁶)</td>
<td>NMRI-foxn-1 (Age&gt; 8weeks)</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>NCI-H661(0.5 x10⁶)</td>
<td>NMRI-foxn-1 (Age&gt; 8weeks)</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>SW900 (3 x 10⁶)</td>
<td>NMRI-foxn-1 (Age&gt; 8weeks)</td>
<td>IV</td>
</tr>
<tr>
<td>Orthotopic/Immunocompetent</td>
<td>BT4C (1 x 10⁶)</td>
<td>Inbread BDIX rat (200-250g)</td>
<td>III</td>
</tr>
</tbody>
</table>

Tumor implantation. Animals were anesthetized (with fentanyl-fluanisone-midazolam) during inoculation of the tumors or viral transductions (I, III, IV). Tumor cells were inoculated in 50µl volume of OptiMEM medium (Gibco, BRL, Life Technologies, UK) subcutaneously or orthotopically to the lungs, with a 27G needle. Tumor cells were injected subcutaneously into two sites of the back of each animal and tumors were grown approximately two weeks before treatment (I, III). Orthotopically implanted lung cancer cells were inoculated with a direct injection through the chest wall at the mid-axillary line at the level of caudal point of scapula (Figure 6.). To standardize the depth of the injection we used stopper that prevented deeper penetration (IV). Rat glioma cells were implanted in a 5 µl volume of OptiMEM intracranially in the right corpus callosum at the depth of 2.5 mm at the following coordinates; 1 mm caudal to bregma and 2 mm right to sutura sagittalis using stereotaxic apparatus (Köpf, Berlin, Germany) (III).
Figure 6. Orthotopic tumor implantation to right lung parenchyma. Method was modulated from that described by McLemore and co-workers (McLemore et al. 1988).

Tumor size assessment. The tumor growth was analyzed with different methods depending on the tumor location. The three dimensional size of the subcutaneous tumors was measured with a ruler (I, III). The growth of the orthotopic lung tumors was monitored with small animal computerized tomography (CT) (X-SPECT®, GammaMedica, Inc.) to define the treatment start point. The imaging was carried out after viral administrations (once a week) and thereafter every second week. During the process, the animals were anesthetized with 4% isoflurane in 70/30 N₂/O₂ in an anaesthesia chamber and when performing the imaging, the isoflurane level was reduced to 1.4% (IV). To monitor the intracranial tumor growth, magnetic resonance imaging (MRI) was carried out using Magnex 4.7 T magnet (Magnex Scientific Ltd, Abington, UK), equipped with actively shielded gradients (Magnex Scientific) interfaced to Varian UNITY® INOVA console (Varian inc., Palo Alto, CA, USA) (III).

Table 6. Treatments with viruses and viral vectors performed in studies I-IV

<table>
<thead>
<tr>
<th>TUMOR MODEL</th>
<th>VIRUS/VECTOR</th>
<th>ADMINISTRATION ROUTES</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous lung cancer</td>
<td>AdTK-GFP + GCV</td>
<td>i.t. (4.8 x 10⁹ pfu in 30µl) + GCV i.p. (50 mg/kg/day for two weeks)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>VA7-EGFP</td>
<td>i.t. (1.7 x 10⁷ in 30µl), i.v. &amp; i.p. (5.8 x 10⁸ in 100µl)</td>
<td>III</td>
</tr>
<tr>
<td>Orthotopic lung cancer</td>
<td>VA7-EGFP</td>
<td>i.t., i.v. (5.8 x 10⁷ in 100µl)</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Ad5-Δ24TK-GFP</td>
<td>i.t., i.v. (5.8 x 10⁷ in 100µl)</td>
<td>IV</td>
</tr>
<tr>
<td>Orthotopic rat glioma</td>
<td>SFV A7 (74)</td>
<td>i.t. (2 x 10⁷ in 20 µl)</td>
<td>III</td>
</tr>
</tbody>
</table>

Gene transfer efficiency. Four days after transduction, two animals were sacrificed and tumors (four) were collected and homogenized mechanically and in enzyme solution (0.002% w/v Dnase I (type IV, Sigma) 0.1% w/v collagenase (type IV, Sigma) and 0.01% w/v hyaluronase (type V,
Sigma). EGFP-positive cells were used to determine the transduction efficiency with flow cytometry (FACSCalibur, Becton Dickinson) (I, III)

**Therapeutic efficacy.** Therapeutic efficacy was analyzed as tumor size reduction (I, III) or as enhanced survival of the animals (III, IV).

### 4.4 ANALYTICAL METHODS

**Table 5. In vitro assays used in studies I-IV.**

<table>
<thead>
<tr>
<th>IN VITRO ASSAY</th>
<th>DESCRIPTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganciclovir sensitivity assay</td>
<td>GCV sensitivity was determined as increasing cytotoxicity with increasing proportion of TK-gene positive cells after GCV exposure. Cell viability was analyzed with MTT assay according to manufacturer’s instructions (Cell proliferation kit II, Roche Diagnostic, Indianapolis, IN).</td>
<td>I, II</td>
</tr>
<tr>
<td>Bystander-effect analysis</td>
<td>Cell populations with 20% of TK-GFP positive cells were seeded with increased cell densities and incubated with 5µg/ml GCV for five days. Cell viability was analyzed by MTT assay. Bystander positive cells display &gt; 20% cell death compared to controls.</td>
<td>I</td>
</tr>
<tr>
<td>Flow cytometric analysis</td>
<td>EGFP expressing cells were used to determine gene transfer efficacy and transgene expression level.</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Luciferase assay from tissue samples</td>
<td>A Lucilite luciferase reporter gene assay kit (Perkin-Elmer life sciences, Boston, MA) was used to analyze the tissue specificity of human hHKII promoter in vivo. The analyzed tissues were excised from three transgenic mouse lines that expressed luciferase gene driven by different sized fragments of hHK II promoter (Pirinen et al., 2004).</td>
<td>II</td>
</tr>
<tr>
<td>Crystal violet staining</td>
<td>Verification of viral replication and oncolysis at different time points. Stains viable cells violet.</td>
<td>IV</td>
</tr>
<tr>
<td>Western Blot analyzes</td>
<td>Analysis of possible type I IFN response in A549 subcutaneous tumors after VA7-EGFP treatment. MxA protein was used as positive marker for type I IFN response.</td>
<td>III</td>
</tr>
<tr>
<td>Analysis of neutralizing antibodies (ab)</td>
<td>Serum samples were diluted and incubated with viral vector for 1 h at +37°C and added onto cells. Appearance of neutralizing ab:s was detected as inhibited replication of viral vector.</td>
<td>III</td>
</tr>
<tr>
<td>Histology</td>
<td>Tumor samples were fixed with paraformaldehyde (PFA) and embedded in paraffin. Four µm sections were stained with haematoxylin-eosine (HE).</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Immunohistology</td>
<td>Tumor samples were fixed with PFA and embedded in paraffin. Four µm sections were stained with rabbit polyclonal SFV antibody or rabbit polyclonal Ad2-E1A antibody coupled with a peroxidase anti-rabbit antibody conjugate.</td>
<td>III, IV</td>
</tr>
</tbody>
</table>
5 RESULTS

5.1 HSV-TK/ GCV SUICIDE GENE THERAPY IS EFFICIENT AGAINST NSCLC IN VITRO AND IN VIVO (I)

In vitro studies. The HSV-TK/GCV approach is one of the most extensively studied methods in cancer gene therapy. We evaluated the efficiency of first generation adenoviral vector in transducing different lung tumor cells. The achieved gene transfer efficacy was very high with all cell lines (I, Table I) ranging from 57.2 % to 87.8 % as indicated by the proportion of TK-GFP positive cells three days after transduction. Ganciclovir sensitivity analysis with lung cancer cell lines showed different responses to treatment (I, Fig. 1). Adenocarcinoma cells appeared to be good targets for this treatment strategy since only 10 % of TK-GFP positive cells and 0.1 - 10 µg/ml concentration of GCV were needed to obtain evident therapy related cell destruction. The adenoviral vector used in this study was toxic to the large cell lung cancer cell lines, since only about 1 % of TK-GFP positive cells was enough to induce cytotoxic effect that killed some 40 % of the cells. Only two cell lines (NCI-H1650 and NCI-H23) were extremely sensitive to GCV (nearly 50 % of the parental cells were destroyed with 10 µg/ml of GCV) and one squamous cell lung cancer cell line (NCI-H520) was completely resistant to this therapy. A good bystander effect is considered a very important feature of the target tumor cells for obtaining sufficient response to HSV-TK/GCV based therapy. It transpired that the adenocarcinoma cell lines displayed a prominent bystander effect, whereas those cells that were resistant to HSV-TK/GCV therapy also had a low level of the bystander effect.

In vivo studies. To further evaluate the utility of HSV-TK/GCV gene therapy in the treatment of NSCLC, we established subcutaneous tumor models, one representing each main NSCLC type (adenocarcinoma, squamous cell lung cancer and large cell lung cancer). The selection of the cell lines (A549, SW900 and NCI-H661) was based on the in vitro studies and preliminary tumor cell implantation studies. Also the number of cells required to induce subcutaneous tumors to NMRI-foxn-1 nude mice flank was determined in the preliminary studies. In vivo gene transfer mean efficiency appeared to be reasonably high for each tumor type, ranging from 13.7 % to 19.2 % (I, Table II). This was thought to be sufficient to provide significant tumor eradication after GCV treatment. All tumor types, when subjected to AdTK-GFP gene transfer and GCV treatment were significantly (p < 0.001) smaller compared to controls (I, Fig. 3). The large cell lung cancer cell – based tumors were the most sensitive to the treatment, displaying almost complete tumor eradication. Because of the rapid proliferation of the large cell lung cancer tumors in the control
groups, this part of the experiment had to be terminated after 10 days of GCV treatment, whereas the other tumor types received 14 days of GCV before the animals were sacrificed. GCV alone did not have any effect on tumor growth and evoked no signs of direct toxicity of GCV. Adenovirus itself had an effect in adenocarcinoma and squamous cell tumors, since the transduction with vector as such caused some decrease in tumor sizes (I, Fig. 3).

5.2 TARGETING PROPERTIES OF HUMAN HEXOKINASE II PROMOTER TO NSCLC CELLS (II)

Tissues from different transgenic mice lines carrying various fragments of hHKII promoter (505, 819 and the full length 4077 bp) driving the expression of luciferase marker genes were analyzed to examine whether the hHKII promoter activity would be low in the normal (non-malignant) lung tissue. The expression of the transgene was low in most organs, but some elevation was detected in the brain (with shorter promoter fragments) and muscle (with full length promoter fragment). Importantly, the expression of the marker gene in the lung was virtually undetectable (II, Fig. 2). To further analyze whether the hHKII promoter was more active in cancer cells (with highly active glucose metabolism) than in normal cells, we created a lentiviral (second generation, pseudotyped with VSV-G) vector that had different fragments (352, 982 and 1820 bp) of hHKII promoter to drive the expression of the TK-GFP fusion gene. The titers of the targeted lentiviral vectors were moderate, ranging from \( 4 \times 10^5 \) to \( 8 \times 10^6 \) transducing units (TU) per ml and all three promoter fragments displayed efficient properties already with low multiplicity of infection (MOI 1) in the tested NSCLC cell lines: strongest expression was gained with the longest fragment and weakest was with the middle sized fragment, which nonetheless achieved a proportion of \(~ 25\%\) of GFP expressing cells (II, Fig. 3). The targeting properties were found to be most favorable with the shortest fragment (352 bp), the transduction efficiency in NSCLC cells being high compared to the low transgene expression in primary human lung fibroblast and lung epithelial cells. Hence, further studies were carried out using only the vector with the best targeting properties (hHKII-352). The utility of hHKII-352 vector to transduce also lung, ovarian, prostate and brain cancer cells was compared to the efficacy of the vector with a ubiquitous EF1\(\alpha\) promoter. Unexpectedly, our new targeted construct was in many cases even stronger in transducing cancer cells when a rather low multiplicity of infection (MOI 1) was used (II, Fig 4). Furthermore, the EF1\(\alpha\) vector was much more active in transducing primary bronchial epithelial cells and primary lung fibroblasts than the hHKII-352 vector (II, Table I). However, the activity (as measured by transgene expression level) of the EF1\(\alpha\)-promoter was somewhat higher in most cell lines (II, Fig. 5), but when the HSV-TK/GCV suicide gene therapy experiment was
performed, it revealed that the functionality of the hHKII-352 promoter was comparable to the EF1α-promoter. This study demonstrated out that the hHKII promoter has potential in transcriptional targeting of viral vectors for cancer gene therapy and further studies are warranted.

5.3 SFV VIROTHERAPY IN SUBCUTANEOUS TUMOR MODEL AND BIODISTRIBUTION OF THE VIRUS (III)

*Efficacy in vitro.* SFV virotherapy was first evaluated for its potential to eradicate tumor cells *in vitro*. The replication competent vector VA7-EGFP (based on avirulent strain of SFV) (Vähä-Koskela et al., 2006) was very rapid and efficient in killing all three main types of NSCLC cells *in vitro*. Detectable cell destruction was observed with flow cytometry and fluorescent microscopy already at 24 hours after infection and by 48 hours post-infection, most of tested NSCLC cells were eliminated and the remaining live cells were expressing EGFP (*Figure 7.*).

![Figure 7.](image)

*Figure 7. In vitro efficacy of VA7-EGFP oncolysis in A549 adenocarcinoma cell line at 48 h post-transduction.* The left panels represents the control A549 cells a) light microscopy image and c) fluorescence microscopy image. The right panels are VA7-EGFP infected (MOI 1) cells b) light microscopy image and d) fluorescence microscopy image.

When crystal violet staining was performed after VA7-EGFP infection, all three NSCLC cells were killed efficiently with MOI 10 in four days. However, with lower MOIs (0.1 and 1) there were some viable adenocarcinoma and squamous cell lung cancer cells left at 10 d post-infection (IV, Fig. 1b). A similar experiment was performed with the conditionally replicative adenovirus
Ad5-Δ24-TK-GFP for comparison. This study revealed that the replication kinetics was three times slower than with VA7-EGFP, since complete cell destruction was seen at 12 days after infection. The large cell lung cancer cell line (NCI-H661) also appeared to be almost resistant to Ad5-Δ24-TK-GFP.

**Oncolytic efficacy in subcutaneous tumor model.** The VA7-EGFP virotherapy has been earlier tested in human melanoma xenografts in SCID mice (Vähä-Koskela et al., 2006). Our purpose was to assess the oncolytic capacity of this novel oncolytic anticancer agent in a more immunocompetent animal; in the nude mouse that lacks only the functional T-cell response but still having the antibody mediated responses as well as other effectors of the immune system, e.g. NK cells. The antitumoral response was clear in locally (i.t.) treated subcutaneous human lung adenocarcinoma tumors, since three weekly injections reduced significantly (p < 0.001) the tumor growth compared to that seen in control tumors’ (III, Fig. 1a &1b). However, the systemic administration of the vector did not support the virus replication within the tumors and the tumor sizes did not differ significantly from that of the control tumors at the end of the study (III, Fig. 1c and 1d). The tumor growth in i.v. treated animals was slightly delayed, but the growth was accelerated towards the end of the treatment period and no differences in the tumor sizes were detected between the treatment groups (control, i.v. and i.p.) at the end of the study.

**VA7-EGFP distribution in immunodeficient mouse tumor model.** The distribution analysis was performed using the same subcutaneous tumor model to clarify the effects of the VA7-vector after different routes of administration (i.v., i.t. and i.p. routes) to nude mice. Based on the earlier study done with SCID mice (Vähä-Koskela et al., 2006) virus positive areas were found mainly in the brain and in the tumors. Therefore, we collected only the serum, the tumors and the brains from mice at 16 h, 48 h, 96 h and one week after virus injections. Only transient viremia was detected from the serum samples during the first 48 hours, irrespective of the route of administration. The tumors were loaded with more viruses when injected i.t., but also some virus positive areas were detected in systemically treated tumors (III, Fig 2). The neurovirulent nature of the SFV virus was shown in the distribution study, since at later time points, the virus was detected only in the brain (III, Table I). Despite this fact, none of the VA7-EGFP treated animals displayed any neurological symptoms during the experiment. The lack of response in systemically treated animals is usually related to unwanted immune responses, such as the type I IFN response of the cells against the virus. We studied the possible type I IFN response with western blot analysis of the MxA indicator protein from the tumors collected at different time points (16 h, 48 h, 96 h and one week post-infection). We detected a prominent type I IFN
response irrespective of the administration route (III, Fig 3). The response was demonstrated to be time-related (after infection) and dependent on the administration route.

5.4 VIROTHERAPY PROLONGS THE SURVIVAL OF NUDE MICE WITH EXPERIMENTAL LUNG CANCER (IV)

One reason for the relatively low response in systemically treated subcutaneous tumors could have been the insufficient blood flow to and within the tumor. The orthotopic lung cancer model was created to provide a more relevant microenvironment for tumor growth and thus have a better vascularization and blood supply within the tumor. The same adenocarcinoma cells (A549) used in the earlier studies induced a highly aggressive lung cancer in nude mice (IV, Fig. 2). To confirm the presence of tumor xenografts in the lungs, we utilized small animal CT, which allowed a precise and straightforward monitoring of both the tumor development and the efficacy of the treatment. In the CT lung scans, a clear response to VA7-EGFP treatment was seen (IV, Fig 3.). The virotherapy in orthotopic lung cancer model was powerful with locally administered naturally oncotropic VA7-EGFP vector, with the engineered conditionally replicative adenoviral vector (Ad5-Δ24-TK-GFP) used for comparison. The overall survival increased significantly with both vectors, but again, we failed to trigger a significant anti-tumor response with systemically administered virotherapy with SFV. Only a modest elevation in median survival was achieved in i.v. treated animals (IV, Fig. 4).

5.5 FAILURE OF SFV VIROTHERAPY IN IMMUNOCOMPETENT RATS (III)

Finally the SFV virotherapy approach was tested in animals with a fully functional immune system to elucidate the possible effect of the intact immune system in the response to treatment. The immunocompetent animals were treated with an avirulent strain of SFV A7(74) (Tuittila et al., 2000) that has been proven to be nearly identical to VA7-EGFP in causing the oncolysis in cancer cells, but which lacks the transgene. The BDIX rat glioma model was used to study the antitumoral effect of SFV virotherapy. One single i.t. injection of the virus retarded the tumor growth for ten days (p < 0.05) but thereafter the tumor growth was accelerated and tumor reached the sizes observed in the tumors of the control animals. Moreover, the survival of the treated animals was not prolonged (III, Fig. 4b and 4c). During the treatment, MRI monitoring revealed that virus treated tumors had centrally located necrotic areas (lower contrast in MRI scans) (III, Fig. 4a), caused presumably by the viral replication within the tumor. The ten day halt in the
tumor growth which was subsequently followed by increased growth rate points to the involvement of immune mediated clearance of the virus. Next, we clarified whether repeated injections or a transient immune suppression (with the clinically relevant T-cell suppressor dexamethasone) could improve the therapeutic outcome of the treatment. Unfortunately, neither approach enhanced the response to treatment (III, Fig. 5). At the end of this study, the serum samples were collected and were used to define whether neutralizing antibodies had been generated against SFV antigens. The in vitro neutralization assay revealed a dramatic inhibition of the VA7-EGFP replication in BT4C cell culture when incubated with diluted SFV treated animals’ serum. The control sera (serum from an untreated animal) did not inhibit the VA7-EGFP replication (III, Fig. 6)
6 DISCUSSION

The NSCLC is one of the deadliest cancers killing approximately 1.2 million people annually worldwide (Parkin et al., 2005). The prognosis is dismal and the conventional treatment forms remain mostly palliative (Roth et al., 2001). Therefore, new therapy modalities are urgently needed to improve the survival and the quality of life of NSCLC patients. Gene therapy and virotherapy are new approaches that can take into account and utilize the differences between the malignant cells and the normal cells. Thus, more targeted and localized effects can be achieved in this way, enhancing the therapeutic index and reducing the unwanted side effects in normal tissues. The HSV-TK /GCV based gene therapy has been one of the most widely tested novel anti-cancer treatment form and its utility against localized tumors such as gliomas has been demonstrated (Immonen et al., 2004). The virotherapy with viruses that replicate in a tumor specific manner has been shown to possess therapeutic potential, even when the viruses are administered systemically, making it possible to reach those tumors that are located in inoperable places or in a cancer that has metastasized (Liu and Kirn, 2007). Due to the lack of efficient treatment for NSCLC, both HSV-TK/GCV gene therapy and virotherapy were evaluated here against different NSCLC models.

6.1 HSV-TK/ GCV GENE THERAPY AGAINST NSCLC

HSV-TK/GCV suicide gene therapy is the oldest (Moolten, 1986) and the most widely used cytotoxic gene therapy modality that is especially suitable for treatment of localized cancers (Albelda et al., 2000). Some studies for evaluating the feasibility of HSV-TK/GCV therapy in lung cancer treatment (Hasegawa et al., 1993; Nishino et al., 2001) have been performed earlier. Here, the efficacy of adenovirus mediated HSV-TK/GCV therapy for different types of NSCLC in vitro and in subcutaneous tumor models was evaluated thoroughly.

The gene transfer efficiency was high with low MOI (3) in all tested NSCLC cells lines in vitro and in vivo. The rate of transduction in vivo (~15%) was promising, considering the final response to treatment, since it has been shown in earlier animal studies that complete regression of the tumor can be achieved with as few as 10% of HSV-TK positive cells (Culver et al., 1992).

Bystander effect. A very important factor in terms of response to HSV-TK/GCV treatment is the bystander effect where neighboring non-transduced cells are also exposed to the toxic GCV-triphosphate (Culver et al., 1992). This is an essential feature in achieving a total response, because it is almost impossible to introduce the therapeutic gene into all cells of the tumor with
the current vectors (Kuriyama et al., 1999a). The cell culture studies showed a clear correlation between the treatment response and the bystander effect in NSCLC cell lines. The GCV sensitivity assay showed that different cell types responded differently to the treatment, with adenocarcinoma being the best target, the squamous cell being more complicated, with one totally resistant cell line and large cell lung cancer cells dying more due to vector toxicity. The adenocarcinoma cells were the most bystander positive cells in this study, as opposed to squamous cell lung cancer cells where we observed one completely HSV-TK resistant and a bystander-negative cell line. The different sensitivities to HSV-TK/GCV therapy and the differences in the bystander effect between cell lines can probably be explained by the extent of gap junctional (intercellular channels that allow the transfer of small ions and molecules) coupling between cells. If cells lack these endogenous channels, they usually do not display a bystander effect and this reduces their sensitivity to therapy (Aghi et al., 2000). Based on the results of the bystander effect and GCV sensitivity assays, we chose one cell line representing each main type of NSCLC for use in the subcutaneous tumor studies.

**Cytotoxicity.** The cytotoxicity caused either by the vector or GCV was evaluated *in vitro* and *in vivo*. *In vitro*, the large cell lung cancer cells were found to be highly sensitive to the Ad-TK-GFP vector, since with 1 % proportion of Ad-TK-GFP positive cells, a 60 % decrease in viability compared to the controls was seen. Interestingly, this was not the case *in vivo*, where the tumor growth did not decrease after injection with Ad-TK-GFP. With respect to the other cell types (adenocarcinoma and squamous cell lung cancer), there was an opposite effect, with some vector toxicity observed *in vivo*. The Ad-TK-GFP vector alone has also been shown in earlier studies to delay the tumor growth (Pasanen et al., 2003; Wiewrodt et al., 2003). Even though the animal models used in these studies were immunodeficient nude mice, the vector related tumor eradication could be explained to some extent by an immune response targeted against the vector or the transgene. The GCV toxicity was detected in two adenocarcinoma cell lines and these were not included in the subsequent studies. High concentrations of GCV have also been shown to be toxic to mice (Zhang et al., 1997), but in this study, no toxicity was detected when using 50 mg/ml/kg divided into two daily injections. It is assumed that, with the two daily injections, the GCV levels of the system remained more stable.

*In vivo efficacy.* The adenoviral natural tropism to the lung cells probably led to successful transduction of NSCLC cells also *in vivo*. As predicted after efficient transduction, the response to treatment was also good, resulting in significant tumor eradication in immunocompromised animals (nude mice) with all tested NSCLC tumors. It has been argued earlier (Kuriyama et al., 1999a) that HSV-TK therapy would be effective only in immunocompetent mice since it requires
T-cell mediated immune response and clearly our study but also the work by others (Kurdow et al., 2002; Wiewrodt et al., 2003) carried out with immunodeficient animals do not support this theory. However, it has been proven that the antitumor immune response mediated through mononuclear macrophages, cytokines and T-cells (CD4+ and CD8+) plays a role in HSV-TK therapy (Vile et al., 1997) and in immunocompetent animals, the response is probably further enhanced by the CTLs. One factor that is believed to have a positive effect in this study was the fact that we started to administer GCV i.p. on the following day after transduction. In this way the drug was present in the animals as soon as the TK-gene was expressed in the tumors and the cytotoxic action was initiated as quickly as possible.

This modality has been studied in phase I trial using adenoviral vector to introduce the HSV-TK gene to mesothelioma. In that trial (21 patients), no beneficial clinical responses were seen throughout the study, probably due to strong anti-adenoviral immune responses (Molnar-Kimber et al., 1998; Sterman et al., 1998). However, in another study there were two long-term (> 6.5 years) survivors after HSV-TK/GCV treatment (Sterman et al., 2005). The same research group has also showed the safety of intrapleural administration of Ad-HSV-TK in primates (Kucharczuk et al., 1996). In summary, adenovirus-mediated HSV-TK/GCV gene therapy appears to be a promising modality for the treatment of lung malignancies.

6.2 TARGETED HSV-TK/ GCV TREATMENT

The transfer of HSV-TK followed by exposure to GCV resulted in efficient killing of NSCLC cells in vitro and in vivo. To avoid toxicity against normal neighboring cells, selective targeting of the suicide gene is required (Katabi et al., 1999; Swisher and Roth, 2000). The targeting of gene therapy increases the therapeutic ratio, but also provides the possibility to deliver systemically the therapeutic gene and in this way to target also metastasis (Pedersen et al., 2005; Robson and Hirst, 2003). The essential issue in targeting is to identify the functional differences in malignant cells that can be used for targeting the treatment only to cancer cells (Robson and Hirst, 2003). The transcriptional control of expression with tumor specific promoters such as hTERT has been used for targeted gene therapy (Kawashima et al., 2004; Song, 2005). Here we used the different microenvironment and the physiology of the cancer cells, such as elevated energy consumption, as a target by using a promoter of human hexokinase II gene (hHKII). The hexokinase enzyme catalyses the first step of glycolysis (Rempel et al., 1996) and is overexpressed in human cancers (Goel et al., 2003).
The analysis of the tissues from transgenic mice expressing luciferase gene under the control of different fragments of hHKII promoter (Pirinen et al., 2004), showed only low activity in most tissues and most importantly in the lung. In humans, the most active hexokinase type in the lung is HK-I (Riddle et al., 2000). Some luciferase activity was detected in typically insulin responsive tissues such as skeletal muscle. Interestingly, the brain tissue seemed to support some luciferase activity with all hHKII promoter fragments even though the HKII expression is believed to be low in the rodent brain (Heikkinen et al., 2000). This indicates that these promoter fragments lack brain-specific suppressor element(s). However, this is only evidence that the human promoter is functional in the mouse brain, but not necessarily address the situation in the human brain.

We inserted different fragments of hHKII promoter in lentiviral vector which have been shown to be almost as efficient in transducing human NSCLC cells in vitro than the adenoviral vectors (Pellinen et al., 2004). The produced hHKII promoter -targeted viruses were functional and even with low MOI, they were able to transduce NSCLC cell lines efficiently. The mid-size (982 bp) fragment was the weakest and the 352 bp almost comparable to the efficacy of the 1820 bp fragment. Probably the promoter region upstream from the nucleotide 982 contains some elements (transcription factor binding sites) that are enhancing the activation via some stimuli present in lung cancer cells. However, this element is not essential for promoter activity since the shortest fragment was able to still drive efficient transgene expression.

When targeting virus vectors to cancer tissue, it is essential to achieve high expression in cancer cells, but a lack of function in normal cells is equally important (Rots et al., 2003; Stoff-Khalili et al., 2005). The shortest fragment of hHKII appeared to have the most favorable features for targeting, with a lack of expression in normal cells and high expression in tumor cells. The longest promoter displayed the highest activity in cancer cells but also in primary cells, which reduced its targeting value.

The activated glucose catabolism in cancer cells is a known feature and indeed also the hHKII expression is induced to maintain the rapid growth of these cells (Goel et al., 2003; Mathupala et al., 1995; Pedersen et al., 2002). The shortest fragment showed activity also in other cancer cell types and this is in line with a previous study using the rat HKII promoter to target virus vectors transcriptionally to NSCLC and also to other cancer cells (Katabi et al., 1999). The authors of this report were even able to stimulate the rHKII by simultaneously adding both glucose and insulin into the medium.
Surprisingly, our results revealed that the efficacy of hHKII-352 was even higher in some cancer cells than the ubiquitous EF1α promoter that was used for comparison. With the EF1α promoter, the expression level was somewhat higher but as demonstrated in GCV-sensitivity study, it did not have any major impact on the outcome and both promoters provided sufficient expression levels for a good treatment efficacy. Therefore, further evaluation of hHKII promoter’s tumor specific activity is warranted, especially \textit{in vivo} with relevant tumor models.

6.3 SFV VIROTHERAPY AGAINST NSCLC

The lack of efficacy in conventional clinical gene therapy trials has generated a need of novel approaches such as virotherapy where the cancer cells are killed as a consequence of viral replication. The beauty of this modality is the selective replication (lack of replication in normal tissues) within tumor tissue that notably increases the therapeutic value of each agent (Kirn, 2000; Norman and Lee, 2005). The selectivity also provides a possibility for systemic administration, making it potentially useful for treatment of highly metastatic cancers, e.g. lung cancer.

We studied the potential of replication selective SFV vector VA7-EGFP to kill human NSCLC cell lines \textit{in vitro} and in different types of tumor models. The oncolysis caused by the VA7-EGFP in NSCLC cell lines was rapid and extensive; killing all the cells with higher MOIs (10), though with lower MOIs, some adenocarcinoma and squamous cell lung cancer cells remained viable (IV, Fig. 1). In the case of A549 cells, it is known that they elicit a strong type I IFN response against viral stimuli (Rautsi et al., 2007) and on the other hand in the case of SFV, the replication has been shown to be inhibited by the type I IFN response (Landis et al., 1998). Therefore, it was hypothesized that in these particular cancer cells, the type I IFN response inhibits the further replication if insufficient amounts of virus are present. One other facet that has been noticed in virotherapy studies is that resistant cells arise from the cancer cell population and this can also be one reason for failure to achieve a total response to virotherapy (Alain et al., 2006; Phuangsab et al., 2001; Vähä-Koskela et al., 2006).

The further evaluation of the SFV-based virotherapy was done in subcutaneous tumor model representing human adenocarcinoma. The weekly-performed intratumoral injections evoked significant growth arrest in the treated group compared to controls. Nonetheless, some cells remained alive and it would have been interesting to see whether continuous weekly injections could have controlled the growth more adequately. A few naturally tumor-specific viruses (NDV and reovirus, both RNA viruses) have also shown potential antitumor activity after systemic
injections (Hirasawa et al., 2003; Phuangsab et al., 2001). The VA7-EGFP virotherapy failed to induce tumor eradication when injected systemically (i.v. or i.p.). The i.v. treated tumors showed some response after three injections but at the end of the experiment, the tumor sizes did not differ significantly between the groups. One explanation for this phenomenon could be dilution of the virus in the body i.e. not enough viruses homed in on the tumors to initiate powerful enough replication. In addition, when viruses are administered systemically, e.g. i.v., they are more vulnerable to inactivation by the inhibiting components present in the blood such as complement (Wakimoto et al., 2002), cells of the reticuloendothelial system and neutralizing antibodies (Fazakerley et al., 1993; Fulci et al., 2006). Especially in the case of nude mice, the IgM type antibodies typically are able to clear the virus from the system (Amor et al., 1996).

A distribution study was carried out, to better understand the results. We collected tumor, serum and brain samples. Previously our collaborators, who determined the overall distribution and tropism of the vector in infected SCID mice, detected positive responses mainly in the tumors and in the brain (Vähä-Koskela et al., 2006). In that study, the SCID mice also suffered from progressing neurological symptoms since they lack an adaptive immune system and could not control the infection. The nude mice, which only lack a functional T-cell response, did not develop any clinical detectable symptoms after the viral treatment in our experiments (III, IV). This is in line with other studies performed with the avirulent SFV A7 (74) and nude mice (Amor et al., 1996; Fazakerley et al., 1993). The virus positive areas found in the tumor sites were much larger when animals were treated locally and that correlated with the response to the treatment. This also supports the theory of incomplete homing of the virus to tumors after systemic administration (III, Fig. 2). After 96 h, the SFV positive foci appeared in the brain and only some virus was found in the tumors. This suggests that the components of nude mice immune system eliminated the virus from the tumors and also the innate defense mechanism (type I IFN response) of A549 cell line partly inhibited its replication. The type I IFN response (studied from tumor tissues with Western blot against MxA protein) was seen irrespective of the administration route, but the induction kinetics differed. Naturally, the SFV homed earlier to locally treated tumors and the induction of the MxA response was detected more rapidly when compared to systemically treated animals (III, Fig. 3). Although the type I IFN response was strong in locally treated tumors, it was not enough to prevent the viral replication completely and this could be the explanation for the failure to achieve total tumor eradication. Many viruses are known to have mechanisms to prevent this antiviral action e.g. encoding proteins that block the PKR activation (adenoviral VA1 and reoviral σ3) (Everts and van der Poel, 2005). It has also been shown that alphaviruses (Sindbis and SFV) could slow down translation to some extent via specific a hairpin
structure allowing replication in the presence of the active PKR (Ventoso et al., 2006). Thus, this could partly explain that the response to treatment in A549 cells was still feasible even in the presence of a strong type I IFN response.

6.4 EXTENDED SURVIVAL AFTER VIROTHERAPY AGAINST THE EXPERIMENTAL LUNG CANCER

The subcutaneous tumor models are easy to set up and sufficient for robust experimentation to test the feasibility of novel cancer treatment modalities. However, possible inadequate blood supply because of unrealistic microenvironment can lead to results with little clinical value (Killion et al., 1998). The orthotopic xenografts mimic better the human pathophysiologic condition including tumor expansion, vascularization and tissue destruction (Liu and Johnston, 2002; March et al., 2001). Especially when testing new therapy approaches to lung cancer, which is often highly invasive and metastatic, the use of an orthotopic model has been recognized to be necessary and the current recommendation is to utilize these models whenever possible (Chen et al., 2005; Mase et al., 2002; Sarkioja et al., 2006; Shi et al., 2005; Stiles et al., 2006). We implanted the human NSCLC cells into the lungs of nude mice in the same manner as McLemore et al. (McLemore et al., 1988), using a direct injection through the chest wall to the lung parenchyma. The method was simple, efficient and aggressive human adenocarcinoma tumors developed that were invasive, eventually infiltrating to mediastinum (IV, Fig. 2). During the survival study, we used small animal CT which permitted monitoring of the development of the tumor, definition of the starting point for treatment and an assessment of the response to treatment non-invasively throughout the study.

When virotherapy was performed locally, both viruses VA7-EGFP (naturally tumor specific) and Ad5-Δ24TK-GFP (engineered to be tumor specific) induced significant improvement in the survival, i.e. both of the vectors possess clinical relevance. Even though the study was done in nude mice, it should predict at least to some extent the responses that would be obtained when treating human cancer patients that usually are immunocompromised (Davis and Fang, 2005; March et al., 2001). Although viruses that disseminate through the blood have potential to yield efficient systemic responses (Liu and Kirn, 2007), we were not able to observe significant positive responses, not even with the SFV vector. The median survival was extended only by a few days in animals that were treated systemically with either of the vectors. Although we were hoping to observe a better response after systemic injections in this tumor model with more abundant blood flow, we failed to detect any significant difference in survival when compared to
controls (IV, Fig. 4). With both vectors, the results proved to be similar to those obtained with SFV virotherapy in the subcutaneous tumor model. The reasons behind the inefficient response are likely to be similar: a type I IFN response against the vector (Landis et al., 1998; Rautsi et al., 2007), neutralizing IgM-class antibodies (Amor et al., 1996; Fazakerley et al., 1993) and the dilution of the virus in the body fluids before it has opportunity to home to the tumor. Many naturally oncotropic RNA viruses replicate only in cancer cells with deficient IFN or PKR responses. The mechanism behind the SFV tumor specificity is still unknown but it is thought to be related to mutations in Ras, PKR and IFN pathways (Vähä-Koskela et al., 2006). The particularly strong type I IFN response in A549 cells could be a fundamental reason for the poor oncolytic activity via the systemic administration route where the virus amount that reaches the tumor remains below the threshold level. Furthermore, the size of the tumor and the amount of the virus particles correlate with the response to treatment (Gröte et al., 2001) and the rather advanced aggressive cancer model used in this study could have been already too large to be treatable, at least by systemic injections of the vector.

6.5 SFV VIROTHERAPY IN IMMUNOCOMPETENT ANIMALS

An even more complex situation is encountered when SFV virotherapy is tested in a fully immunocompetent animal. The immune system plays a critical role in the efficacy of virotherapy and in some cases this can be truly beneficial by triggering antitumoral immune responses (Schirrmacher et al., 2000; Termeer et al., 2000). The BT4C rat glioma model was used to evaluate the anticancer capacity of replication competent avirulent strain of SFV. In this study, we used the attenuated virus SFV A7(74) without the transgene to prevent the possible immune response against the virus, since it has been shown that T-cell response can arise against the EGFP (Stripecke et al., 1999). In earlier studies SFV A7 (74) and the derived vector VA7-EGFP have been shown to have a comparable tropism, replication kinetics and cytotoxicity (Vähä-Koskela et al., 2003). The main study was performed with local injections, since the systemic injections had failed in preliminary study and in the previous studies (III, IV), using immunologically less competent animal models. A single i.t. injection exhibited an apparent antitumor effect during the first 10 days of the treatment, since the tumor growth was significantly retarded compared to controls (p < 0.05). After this time point, however, the tumor growth was accelerated and at the end of the study there was no significant difference between the treated and the control animals. On the other hand, in the MRI scans of the tumors, lower contrast areas were observed in the central parts of the treated tumors, indicating necrosis attributable to the SFV replication within the tumors. To further investigate the possibility of immune mediated
clearance, we repeated the study and performed the viral injections three times (one injection every third day) or used a pharmacological T-cell suppression (dexamethasone) combined to one injection. T-cell targeted immune suppressants combined to oncolytic therapy could be one possible solution to enhance efficacy and most of these compounds such as cyclosporine, tacrolimus and dexamethasone, have already been evaluated clinically and shown to be safe. (Davis and Fang, 2005). Many pre-clinical studies combining immune suppression with virotherapy have shown enhancement in efficacy (Fulci et al., 2006; Hirasawa et al., 2003; Ikeda et al., 1999; Smakman et al., 2006; Wakimoto et al., 2002). However, despite the multiple injections and the immune suppression, we were not able to achieve any improvement in the response (III, Fig. 5). Therefore, it seems that the T-cells are not playing a major role in inhibiting the SFV replication. On the other hand, we did not verify experimentally whether the T-cell suppression with dexamethasone was actually successful. Thus an insufficient immune suppression rather than the lack of role of T-cells may be the true explanation for the result seen in this experiment. It would be worth examining, whether some other immunosuppressive compound, such as CPA or cyclosporine, would work better in this setting and would enhance the therapeutic outcome.

No virus was detected in the serum or the brain of any of the animals after the treatment. Therefore, it is apparent that the virus had been eliminated through different mechanisms, most probably by neutralizing antibodies, since tumor growth was reinitiated at day 10, which is precisely the time when the virus-specific IgG start to appear. Notably, it has been shown earlier that especially the IgG type antibody is able to clear the SFV A7(74) virus from the brain (cf. IgM or other functional components of the nude mice system were not able to clear the virus from brain) (Amor et al., 1996; Fazakerley et al., 1993). Indeed, the results from the neutralizing antibody assay showed that the VA7-EGFP replication was totally inhibited after incubation with all of the diluted serum samples from SFV treated rats (III, Fig. 6). The actual antibody titer of the rat serum was shown to be even higher than that obtained after the positive control polyclonal anti-SFV anti-rabbit serum. Also, other factors such as injecting the viruses into necrotic core of the tumor can prevent the efficient replication of the virus (Wein et al., 2003). This may have influenced the results of our study, since it became apparent that after one injection, the replication caused necrosis in the center of the tumor and the second injection likely has focused on this area that does not support further virus replication. Therefore, this could be yet another explanation for the low response with multiple injections, though it is more likely that the neutralizing antibodies were the major reason for failure of SFV virotherapy.
Virotherapy is a promising anti-tumor modality, but many aspects have to be carefully taken into consideration, because several factors may affect the outcome of the therapy. The replication competent SFV has shown efficacy in eradicating tumors from SCID mouse after systemic injections (Vähä-Koskela et al., 2006), from nude mouse when administered locally (III, IV) but in the fully immunocompetent rodent model, the therapy failed (III). This suggests that the major barrier for SFV virotherapy is the host immune system. Many virotherapy studies have focused in immune evasion and this seems highly prudent also from our perspective. Many of the systemic therapies have failed because of the immune system (Kelly and Russell, 2007; Liu and Kirn, 2007) even though in some cases, the immune system is considered as the key player (Kim et al., 2006). Failure with one tumor type in one study does not necessarily mean that SFV virotherapy should be discarded as a potential anti-cancer modality. On the contrary, the recent results can be considered as rather promising and warrant further studies with different cancer models. It is also likely that gene therapy (and virotherapy) approaches will be most useful in a multimodality setting rather than as a single agent therapy (Smythe, 2002) and this should also be taken into consideration when designing new pre- and clinical trials.

It has been now some eleven years ago since the first clinical trial with an oncolytic virus occurred. By the year 2005, more than half of the clinical gene therapy trials were carried out using oncolytic viruses. Safety has been unequivocally proven with these agents, but the efficacy especially when administered systemically, has been modest. The ideal anticancer agent that kills specifically also secondary tumors is yet to emerge but some response has been seen even systemically with a few vaccine strains. Furthermore, when combining current conventional therapies to these new approaches, we will hopefully devise an efficient method to eradicate some of the cancers which today are untreated. However, as cancer is known to be a genetically and phenotypically very heterogeneous disease with a highly transforming nature, there is a constant need for new, better and more adaptive treatment modalities, and so this field of research remains challenging.
7 SUMMARY AND CONCLUSIONS

This study was carried out to investigate two new therapies against NSCLC, the disease which lacks efficient curative treatment. The evaluation of the efficacy and safety was done pre-clinically and progressed systematically from \textit{in vitro} experiments to advanced rodent tumor models. The main observations were:

1. Adenoviral HSV-TK/GCV therapy was efficient in all three main types of NSCLC cell lines \textit{in vitro} and \textit{in vivo}. The response to treatment differed between the tested cell lines, emphasizing the heterogenic character of cancer and how difficult it is to find a large scale treatment method that would be effective against all cancer cell types. A positive response was observed only when the cells displayed a detectable bystander effect and cell lines lacking this feature were not suitable targets for this method. The positive results obtained here warrant more studies with more complicated animal models such as orthotopic tumor models and animals with functional immune system.

2. Human hexokinase II promoter was shown to be a strong promoter for transcriptional targeting of the HSV-TK/GCV therapy to human cancer cells. The shortest fragment (352 bp) of the promoter appeared to possess favorable features for targeting due to its strong expression in many human tumor cell lines, but very low expression in human primary cells. The transcriptional control of the therapeutic gene improves the safety and provides an opportunity for systemic administration. These results encourage characterizing further the targeting properties of hHKII-352 in animal tumor models and primary tumor material.

3. Virotherapy with attenuated VA7-EGFP appeared to be safe and efficient in immunocompromised animals. This study demonstrated that locally performed virotherapy can induce efficient tumor eradication and prolonged survival in experimental lung cancer models. The innate barriers that are faced with the systemic administration routes will be challenges in virotherapy. As seen also in this study, both viruses (Ad5-\Delta24-TK-GFP and VA7-EGFP) failed to induce long lasting anticancer effects when administered systemically. However, further studies need to be conducted with other lung cancer cell lines (for example, cell lines lacking the type I IFN response), since the cell specific defense mechanisms play a major role in the success of virotherapy, especially with SFV.
4. SFV virotherapy in immunocompetent animals was shown to be safe, but the virus was vulnerable to the host immune system and was eliminated before any significant antitumoral response was generated. The immune system and its role will be the key issue when attempting to achieve a powerful response with virotherapy in immunocompetent animals. However, failure in one study with one model does not invariably mean that the therapy will not work in another tumor model. Finally, the most promising approach in the future could be the use of combination therapies, exploiting the efficacy and synergy of two different modalities (conventional therapies and virotherapy).


