Gene Therapy and the Evolution of Cancer Treatment

The Main Step in Prevention of the Highest Stages of Cancers And Decreasing Mortality due to Tumors

James Douglas

2007

Abstract: The continued understanding of gene structure, function and how its product interacts in the human body has lead to new wave of ideas on cancer therapy. Gene therapy, which 20 years ago was just a dream is now a very real possibility and alternative to the conventional treatments. Three new methods of utilizing alternative gene structure and manipulating the body's response to induce a tumor effect are presented here. Immunotherapy is the induction of the human immune system against an unwanted antigen. Viral oncolysis is the utilization and manipulation of natural human pathogens against tumor cells. Gene transfer is the addition of deletion of vital genes responsible for cell growth and apoptosis.

If the target of the World Health Organization (WHO) Health 21 to decrease the mortality due to cancer of all sites by at least 15% and lung cancer by 25% by 2020 is to be met, a rash of new treatment options is needed[1]. Five year survival rates for pancreatic (4%), lung (15%), liver (7%) and gliobastoma (5%) remain horribly low[2]. Even most stage III and IV cancers have low to very low 5 year survival, along with the increase in disease and treatment associated suffering.

The treatment options available are surgical removal, radiotherapy, chemotherapy and hormone therapy in certain hormone responsive tumors. Each of these is not without significant side effects. Surgical removal is the best treatment option regardless of tumor state. However, is the case that the tumor has metastatic lesions, surgery is not indicated. Furthermore, surgery is limited to tissue amendable or non vital to surgical remove. Radiotherapy causes fatigue, skin problems, hair loss, bleeding problems and infections, loss of appetite or interference with eating, digesting, and absorbing food, local problems related to area of exposure and post treatment secondary neoplastic disease. Chemotherapy has many systemic effects. While not as severe as it once was, patients still suffer from fatigue, nausea and vomiting, pain, anemia, infections, blood clotting problems, diarrhea or constipation, a peripheral neuropathy, fluid retention, loss of sexuality and many other potential problems. Hormonal therapy is a temporary treatment for certain hormonally responsive tumors. By suppressing the driving mechanism, one can decrease the hormonally driven growth of tissues. This is temporary however as all tumors eventually become hormone independent. Furthermore patients treated by hormone treatment or ablation suffer from many symptoms related to the hormone.

The commonality of these treatments is that they don't attack the root, only attempt to remove the problem. The newest lines of evolving treatment possibilities are

much more specific in their intent. Gene therapy is in essence the modification of cells to affect a cure. This can either be cells responsible for the uncontrollable growth or cells responsible for detecting and removing irresponsible cells. There is naturally in any newly evolving field, especially with genetic material, high concerns. However to date the side effect profiles of early studies suggest that the worse effects are similar to the common cold: fever, arthralgia, myalgias and pain at the injection site. The field of gene therapy in cancer treatment has been broken down into three broad categories: immunotherapy, oncolytic virotherapy and gene transfer.

Immunotherapy

Clearly there has been evidence that normal human immune defenses are prepared to deal with tumor growth. Inevitably that response is too little to control the mounting tumorous growth. With rapidly expanding knowledge of the immune mechanisms at work can we begin to understand the balance between normal and abnormal tissue, and start to play with possibilities of manipulating that balance. The major histocompatibility complex (HLA molecules) sample proteins and express them on the surface of cells for exposure to T cells. The MHC class I molecules (HLA A,B,C) are specific in that they sample all proteins produced inside the cell, and express they on the surface along with MHC class I complex to CD 8+ T lymphocytes. Via this pathway are viral infected cells and tumor antigens recognized. Immunotherapy identifies the specific antigens presented on tumor cells and adjusts the immune response to attack specifically those cells. Although there is clear evidence of T cell responses, the latest trials are manipulating the antigen presenting cells or the T cells to upregulate the response.

Various cancer vaccine modalities exist:

1. **Modified tumor cell vaccines**

Although the ideal source of antigens comes from the specific tumor itself, the ability to process and produce individualized vaccines is not possible at this time. Allogenic or even generic cell lines are more available and can be modified to upregulate either the antigen expression or the immune response. The most effective modification to allogenic vaccines have been via co-expression of GM-CSF, a cytokine known to increase dendritic cells and other antigen presenting cells at the site of expression. GVAX (granulocyte-macrophage colony-stimulating factor [GM-CSF] gene transduced irradiated prostate cancer vaccine cells) is currently in Phase III testing after showing good results in early studies and almost total tumor cell eradication in murine models[3]. A metastatic malignant melanoma study using recombinant human GM-CSF (rhGM-CSF) injected with autologous melanoma vaccine demonstrated tumor significant tumor regression. Twenty stage IV melanoma patients were treated as outpatients with multiple cycles of autologous melanoma vaccine and bacillus Calmette-Guerin (BCG) plus recombinant human GM-CSF injection in the vaccine sites. Two patients (10%) showed a complete response, with one patient showing resolution of subcutaneous, hepatic, and splenic metastases. In the second patient, buccal, subcutaneous, pulmonary, paraaortic, hepatic, splenic, and retroperitoneal metastases regressed completely. Two patients (10%) showed partial response, with regression of a paraaortic metastasis in one patient[4]. Another phase I trial in patients with pancreatic cancer using GM-CSF transduced

allogenic pancreatic cancer cell lines, 3 out of 14 patients remained disease free at 23 months.

A number of other genetically altered autologous and allogenic tumor cell vaccines expressing IL-2, IL-4, B7.1 and alpha-(1,3) galactosyltransferase are currently in clinical trials.

Malignancy	Vaccine	Upregulator	Phase
NSCLC	Autologous, DNP-Modified NSCLC Vaccine	DNP (dinitrophenyl)	I/II
Melanoma	Autologous, DNP-modified vaccine	DNP	I/II
Mantle Cell Lymphoma	Autologous	GM-CSF, CD40L	II
Renal Cell Carcinoma	Autologous	GM-CSF	I

2. Peptide Vaccines

The elucidation of the amino acid sequence and structure of tumor epitopes, has allowed the possibility to use tumor associated antigens as therapeutic agents themselves. It also required knowledge of the structure of the corresponding MHC molecules and how these short peptides are expressed to the immune system. The advantages of using peptides is that they are easy to produce and stable, can be combined in various peptide cocktails, the response limited to specific epitopes and the epitopes can be enhanced. The enhancement of the epitopes is generally done to improve the affinity for MHC molecules and TCR triggering. The disadvantages of peptide vaccines are that detailed knowledge of the specific epitope is needed, the immunogenic response is limited to a few MHC molecules and usually an adjuvant is needed for adequate reaction[5]. However as the field of tumor antigens is evolving so is the possibility to match HLA types to specific antigens, such is the case with the finding of the new SART antigens[6].

The adjuvant enhancement of the peptide vaccines uses cytokines, chemokines or costimulatory molecules or a combination thereof. Both CD4+ and CD8+ T cells which are the most responsible for the antitumor response are induced into action better by antigen presenting cells like dendritic cells. Therefore it is advantageous to include cytokines like GM-CSF that recruit dendritic cells or cytokines IL-12 or IL-15 that increase the Th1 cytotoxic T lymphocyte response. Another approach uses dendritic cell/T cell costimulatory molecules that increase DC maturation such as CD40L or addition of a CpG-oligodeoxynucleotide, characteristic of bacterial DNA, to elicit a larger response.

The most expansive studies with peptide vaccines have come in malignant melanoma. One study demonstrated a significant prolongation of survival in patients treated with peptide immunotherapy after surgical resection[7]. Epitope enhanced gp100 peptide generated a strong T cell response in 10 of 11 patients immunized. However, only a single objective clinical response was reported[8]. Strangely when immunization was combined with IL-2, the circulating immune precursors dropped and yet 6 of the 16 patients (38%) that received peptide plus IL-2 had objective cancer regressions. The

authors hypothesized that immunization with peptide plus IL-2 resulted in sequestering or apoptotic destruction of newly activated immune cells at the tumor site[9]. Problems with maintaining the immune response has plagued early trials. New studies using immunization with a modified gp100 melanoma peptide in Incomplete Freund's Adjuvant (IFA) results in the generation of antipeptide and antitumor lymphocytes in the patients' circulation that persists for between 138 and 303 days post immunization.

Malignancy	Vaccine	Enhancer	Phase
CML	BCR-ABL junction specific peptide vaccine		II
Melanoma	MDX-1379 (gp100 peptide)	Anti-CTLA antibodies	III
AML, CML	WT-1	GM-CSF	III

3. DNA Vaccines

Numerous strategies exist for DNA delivery systems. The potent therapeutics includes plasmids containing transgenes, oligonucleotides, aptamers, ribozymes, DNAzymes and small interfering RNAs. These strategies combine the penultimate specificity and the lowest negative side effect potentials. Although promise is there, the limited trials so far have met with limited success. This lies in the poor cellular uptake, poor biological stability and short half life. Over the past couple years the understanding of DNA uptake, trafficking and metabolism have been enhanced. Immunotherapy utilizes plasmids for delivery of a tumor antigen and possibly other enhancers directly into antigen presenting cells which can then process the new antigen, express it to T cells and begin the immune surveillance.

Plasmids are high molecular weight, double-stranded DNA constructs containing transgenes, which encode specific proteins. Upon cellular internalization, they employ the DNA transcription and translation apparatus to biosynthesize their proteins. Various immunogenic genes can be incorporated into the plasmid, often along with immunologic up-regulators like GM-CSF. Alternatively other tumor-suppressor, apoptotic genes or non cancer related genes can be added. This will be discussed under gene transfer therapies. One trial involved a plasmid expressing PSA, alone or in combination with plasmids coding for GM-CSF and/or IL-2. The response was evaluated by Cr-release, intracellular IFN-gamma cytokine staining, and tumor challenge assays. The results showed that the DNA vaccine induces PSA-specific cytotoxic T lymphocytes (CTLs) and when co-injected with IL-2 and GM-CSF it protected four of five mice against a PSA-expressing tumor challenge[10].

Malignancy	Vaccine	Enhancer	Phase
Melanoma	gp100 plasmid DNA vaccine		I
Cervical Carcinoma	pNGVL4a-Sig/E7	HSP 70	II
Breast	DNA Plasmid Based Vaccine Encoding the HER-	GM-CSF	I

	2/Neu (HER2) Intracellular Domain		

4. Dendritic Cell Vaccines

Dendritic cells (DC) are the most professional antigen presenting cells (APC) in the body. That is they induce the strongest immune response when presented with a foreign antigen. They exist free in the plasma, however are present in much higher quantities in specific immune response areas such as the intestinal mucosa (MALT) and secondary lymph tissue after migration from the bone marrow.

Immature dendritic cells arise from two precursors: CD 34+ hematopoietic stem cells and CD 14+ monocytes. They mature when presented with the foreign stimuli, in combination with inflammatory cytokines GM-CSF and IL-4, CD 40L or MCM (monocyte conditional media) and possibly other unknown markers. Immature DCs are characterized molecularly by cell surface markers CD1a and HLA-DR. Upon maturation via antigen (Ag) uptake, they up-regulate expression of HLA-DR and express adhesion proteins CD 80, 86 and 83. Once maturated and migrated to areas of lymphatic response, they are capable of inducing a MHC class I response via CD 8+ cytotoxic T lymphocytes (CTL) by a process called priming. Priming is made possible by the corresponding receptors TCR and CD 28 (CTLA-4) and CD 40L. CTLA-4 competes with CD 28 for binding to CD 80/86. It is largely up-regulated upon T cell activation as a measure of immune response regulation. Various methods have been employed to down-regulate the CTLA-4 response with varying success[11,12]. Monoclonal antibodies have been employed in the blockage of CTLA-4 alone and in combination with various blocks of CD 25+ T regulatory cells and GM-CSF[13]. The blockage of CTLA-4 has proven advantageous, however the complete role of the CD25+ T cells is unclear and needs further understanding[14]. Large quantities of IL-12 are produced upon priming, with the subsequent T cell release of IFN-γ upon differentiation into T_H1 cells. IFN-γ is also a potent activator of not only CTL's, but Natural Killer cells (NK cells). Current opinion is that the CD34+ hematopoietic stem cell derived dendritic cell possess a stronger potential for T cell activation over longer durations[15], however they are present in such low quantities compared with the monocyte derived DCs which becomes a deciding factor when quantities required for optimal dosing are considered. New data has shown the addition the bone marrow activating ligand Flt3, can lead to a 10-30 fold increase in circulating DCs. Whether this is sufficient for CD34+ hematopoietic stem cell use, and the DCs prove to be sufficiently active for prostate cancer remains to be known[16].

Delivery of Ag to DCs can be achieved via MHC directed peptides, proteins, carbohydrates, cDNA, RNA, transfection with viruses or plasmids or combinations thereof. RNA is easier to generate than protein, there is little risk of genome integration and multiple antigenic epitopes are expressed. Recently it has been shown that Ag delivery via whole tumor mRNA provides a newer and potentially better approach to vaccine delivery[17]. Whole tumor RNA have the advantage of exposing multiple tumor cell Ags that are unknown, utilizing both a MHC class I and II approach[18] which is needed for the generation of long term CD8+ T cell memory and avoiding the unnecessary HLA haplotyping of the patient. Any fear of generating an autoimmune

reaction have not come to volition, yet cannot be ruled out with such an approach. This method would also decrease the likelihood of tumor escape.

Clinical trials of antigen-pulsed DCs have been conducted in patients with various types of cancer, including non-Hodgkin lymphoma, multiple myeloma, prostate cancer, renal cell carcinoma, malignant melanoma, colorectal cancer, and non-small cell lung cancer. These studies have shown that antigen-loaded DC vaccination is safe and promising for the treatment of cancer. Many clinical trials are underway in different cancers with mixed results. One case from India detailed a complete response in a lady with metastatic gallbladder cancer[19]. Another study found significant results in mice with DC treatment with pancreatic tumor antigens and IL-23 cDNA[20]. A renal cell carcinoma study using dendritic cells cultured with GM-CSF, IL-4 and pulsed with autologous renal tumor cell lysate. The prepared T lymphocytes were cultured with interferon-gamma (IFN-gamma), IL-2, CD3-moAb, and IL-1alpha to prepare cytokine induced killer T cells (CIKs). In 4 patients with measurable disease that received the treatment, 1 had a partial response, 2 had stable disease and 1 had a progressing disease[21]. Liso et al. demonstrated the maintenance of remission in patients with multiple myeloma after high-dose chemotherapy and peripheral blood progenitor cell transplantation (PBPCT) and continued treatment with dendritic cells vaccinated with the Id protein of myeloma immunoglobulin[22]. A study in malignant melanoma using multiple antigens has shown increased infected. The DCs were pulsed with peptides derived from four melanoma antigens [(MelAgs) MelanA/MART-1, tyrosinase, MAGE-3, and gp100], 9 of 10 patients who responded to 2 or more of the 4 antigens had non-progressive disease and regression of at least 1 metastatic lesion[23]. A prostate cancer trial using mouse prostatic acid phosphatase (PAP) xenoantigen loaded DCs, had 6 of 21 patients have clinical stabilization of their previously progressing prostate cancer[24].
Chen et al. also noted a superior combination effect of dendritic cell therapy after pretreatment by radiation[25].

Obviously the ideal goal of any vaccine administration is prophylactic disease prevention. Already two such vaccinations are in circulation: the Human Papilloma virus vaccine for cervical cancer and Hepatitis B virus vaccine for liver carcinoma. Recently another trial was started against gastrointestinal tumor in patients with familial adenomatosis polyposis. These patients have a mutation in adenomatosis polyposis coli (APC) gene and are predisposed to the development of 100's to 1000's of GI adenomas and the inevitable development of carcinoma. This trial utilized the immunization with dendritic cells loaded with syngenic tumor cells (DC/Ts) in mice models. Treatment with DC/Ts prevented the development of gastrointestinal tumors, and coadministration of DC/Ts and IL-12 caused a further reduction in tumor incidence. IgG from the treated mice exhibited cytotoxic activity against the tumor cells in vitro.

Manipulation of the mechanisms of immune response remains the penultimate goal for use in generating an anti-tumor response. Before this can happen, we must clarify the best source of dendritic cells, the dosing regimen, the route of administration and which particular enhancement and homing elements should be used. Lastly a consensus must be reached on clinical outcomes in order to objectify the results from different labs.

Malignancy	Vaccine	Phase

Prostate	Autologous	I/II
Melanoma	Allogenic	I/II
Head and Neck	p53	I
NSCLC	Allogenic	I

5. Recombinant Viral Vectors

Vectors can be programmed to transport various immunogenic molecules to tumor cells. The most popular vector is the adenovirus. Various chemokines and cytokines have proven to increase the immunogenicity such as CC chemokine ligand 16 and ILC/CCL27, IFN gamma inducible protein 10. All have demonstrated an increase in tumor specific T cell responses in animal models. Although an immune response has been demonstrated, there is not enough to cause any significant tumor regression, Many authors speculate this is due to the normally low immunogenicity of the tumor environment. It is still felt that adenoviral transfer of cytokines IL-12, GM-CSF and TNF alpha do boost the immune response and are used in various other gene therapy treatment modalities. Recently a number of studies proved that an intratumoral injection of an adenoviral vector encoding IL-2 in patients with prostate cancer and metastatic melanoma both led to a stabilization of disease[26].

As a modality in itself, the use of recombinant viral vectors as a primary tool for the immunologic treatment of cancer is viewed with much doubt. What however is not doubted in the excellent use of adenoviruses as vectors and the importance of certain cytokines and chemokines to modulate a desired response. Most immunotherapeutic clinical trials are using this technique for delivery to dendritic cells and in the other fields of oncolytic virotherapy and gene transfer.

Oncolytic Virotherapy

Renewed is the interest in using viruses as a treatment option in oncology. It has long been known that viruses can cause tumor cell regression. It was first noted after the turn of the 20th century that a patient with cervical carcinoma experienced tumor regression following rabies vaccination. Furthermore, there have been reports of remissions of Burkitt's and Hodgkin's lymphomas following a measles infection. New interest has sprung back into this field with the evolution of genetic engineering and the manipulation of viruses to narrow their range and focus their destructive mechanisms. The virus itself is a perfectly designed tool for the selective identification and destruction of eukaryotic cells.

Two contrasting methods exist in viral oncolysis and general virology also. Firstly viruses can infect cells and destroy cells by replicating and bursting the cell with many new progeny. Secondly, viruses can be used as vectors in the transfer of certain genes to a cell to induce favorable oncotherapeutic responses. It is of the former that we shall focus here.

The ability of a virus to selectively infect cells of interest is known as cellular tropism of a virus. The virus does this by two main mechanisms. It firstly possesses cell surface receptors that can identify and attach to their counterpart on the cell type of interest. Secondly, once entered into the cell, the virus alters it's phenotype to manipulate the cell to maximize viral replication. How is viral replication modified to specifically infect neoplastic cells? The are 2 mechanisms used to achieve tumor specificity. Firstly there is the deletion of genes necessary for viral replication in non neoplastic cells, but dispensable in neoplastic cells. We can do this by targeting the unique mutations that exist in neoplastic cells like the mutations in tumor suppressor genes like p53 and pRb leading to a loss of cell cycle control. Secondly, tumor or tissue specific promoters can be inserted upstream of viral genes, and be activated during viral replication.

There exists two types of viruses used in trials for oncologic treatment. There are viruses with inherent tumor selectivity, such as the Newcastle disease virus (NDV) and engineered viruses that express certain proteins.

1. **Naturally Occurring Viruses with Inherent Tumoricidal Activity**
    a) NDV- Newcastle Disease virus is a paramyxovirus that infects chickens. It was first reported to have oncolytic abilities in the 1950s. It acts via activation of the mitochondrial death pathway. Two complete responses and 6 partial responses were reported for patients in the treatment group with NDV, whereas no responses were observed in the placebo group. In the treatment group, 10 patients were reported to have stable disease, compared with just 2 patients in the placebo group. In addition, more patients in the treatment group than in the placebo group reported subjective improvements in their quality of life. Twenty-two (67%) of the patients in the treatment group survived at least 1 year, compared with 4 (15%) of the patients in the placebo group. The 2-year survival proportions were 21% and 0% for patients in the treatment group and the placebo group, respectively[27].
    b) reovirus- reovirus multiplicate preferentially in tumor cells with activated gene of ras family or ras-signaling pathway while sparing normal cells. Activated ras or its pathway could be found in as many as 60-80% of human malignancies[28].
    c) Autonomous parvovirus
    d) VSV – The has been some reports in cancer patients, typically children or adolescents suffering from hematologic malignancies, or tumor loads decreasing during episodes of chicken pox or herpes zoster. Few studies are being down right now due to the difficulty controlling herpes infection and the general unpleasantness of it.

2. **Engineered Tumor selective viruses**

A. Adenovirus

The most widely studied virus for the purpose of viral oncotherapy has been the adenovirus. The adenovirus is known to replicate in endothelial cells and has been

considered as early as the 1950's for carcinoma treatment. The adenovirus can serve as a vector for gene therapy permitting gene delivery of DNA up to 37 kb and do not integrate into the host genome thereby providing a safety advantage[29]. They are also stable and relatively easy to manufacture compared to other commonly used viral vectors. When an adenovirus infects a cell, it binds to and upregulates the expression of cellular p53 levels leading to cell replication arrest or apoptosis. Since this is not advantageous for the viral propagation, the virus eludes this by using the protein E1B, which binds to and inactivates p53 and thereby permitting the cellular machinery to proceed. Numerous studies have involved the use of ONYX-015, an adenovirus with a mutation in the E1B gene. Early reports indicated that ONYX-015 only replicated in cells lacking a functional p53, which some estimate involved 50% of tumors. Further tests reveal that ONYX-015 does replicate in cells with functioning p53[30]. Further studies have proven that the target is p14ARF, a tumor suppressor gene that stabilizes p53.

Another technique has involved mutation of the E1A gene, to enhance selectivity for cells with mutations in the pRb pathway. pRb is the last step in cellular progression towards the cell cycle. Early testing was focused on ovarian cancer and demonstrated a decreased activity of HER2 activity following E1A mutated adenovirus administration. E1A gene transfer was demonstrated in 14 of 15 tumor samples tested, and down-regulation of HER-2/neu was demonstrated in two of the five patients who overexpressed HER-2/neu at baseline. HER-2/neu could not be assessed in other posttreatment tumor samples because of extensive necrosis. In one breast cancer patient, no pathological evidence of tumor was found on biopsy of the treated tumor site at week 12. In 16 patients valuable for tumor response, 2 had minor responses, 8 had stable disease, and 6 had progressive disease. E1A mutants have been found to show greater potency that the E1B mutant ONYX-015 viruses both in vitro and in vivo, however these vectors have also been proven to replicate in normal proliferating tissue and therefore will probably have to rely on local delivery to minimize exposures to normal tissue[31].

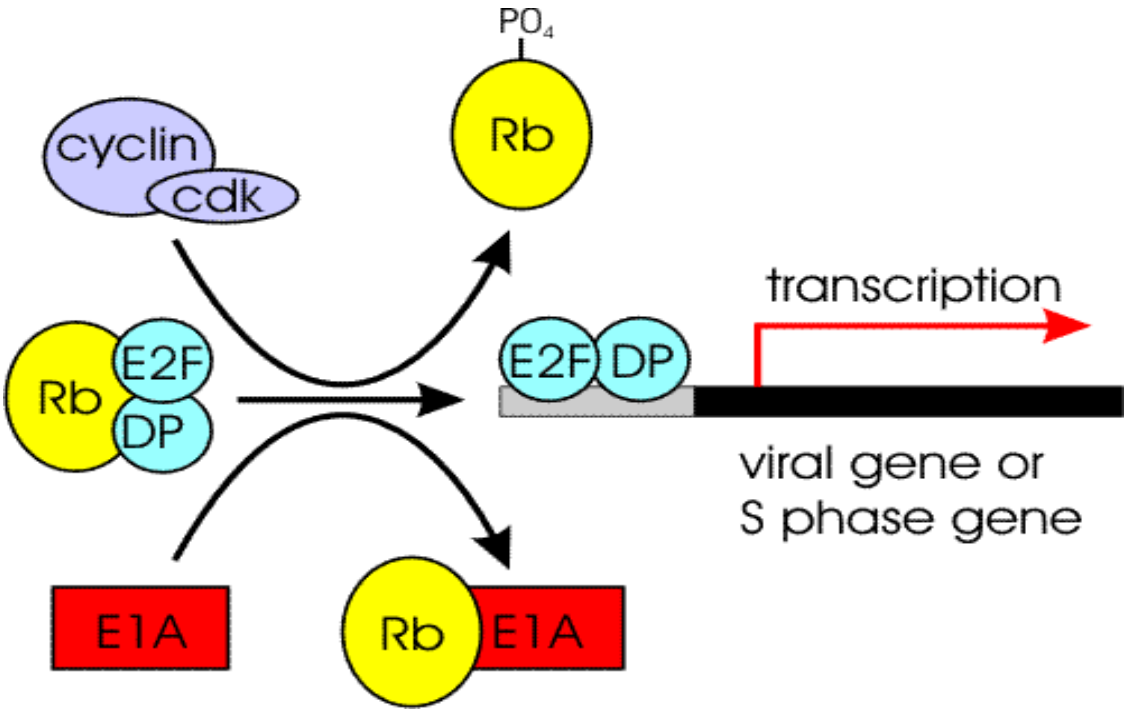

Figure 1. Effect of E1A on pRb/E2F function. In the G0/G1 phase of the cell cycle, hypo-phosphorylated pRb complexes with transcription factors of the E2F family (indicated as E2F and DP) preventing their ability to activate transcription. Cell cycle dependent phosphorylation of pRb by cyclin/cdk complexes releases E2F to activate transcription of target genes required for S-phase of the cell cycle. Expression of E1a overrides the normal cellular control of the pRb-E2F interaction by binding hypo-phosphorylated pRb and freeing E2F. Viral delivery has been tested for safety via intratumoral, intravenous and intra-arterial routes[32].

Various other methods are being utilized to help up-regulate the viral response in tumor cells. The interferon pathway for example is activated via protein kinase R (PKR) in cells infected with viruses, which in turn makes interferon and inhibits viral replication. The adenovirus skirts this method by producing viral-associated RNAs that bind PKR and inhibit its activity[33]. What is also interesting is that some tumor cells that produce the RAS oncogene can also inhibit PKR. Therefore if a virus was created with a mutation in the viral-associated RNA coding region and unable to inhibit PKR, but can selectively replicate only in tumors expressing RAS, it would become a useful oncolytic therapy[34]. Problems have arisen with the low replicating ability of viruses with certain deletions. Cascallo et al. noted a 100 fold decrease in viral replication in viruses with viral-associated RNA coding regions deletions making these viruses unable to kill tumors effectively[35]. Also the E1B protein that inhibit the p53 pathway participates in the nuclear export of late mRNA transcripts and therefore a mutation in the E1B protein impairs replication[36]. Further modification will have to be made or much higher titers of viruses given if a significant therapeutic effect is to be expected.

For the most part, early oncolytic adenoviruses like ONYX-015 have been replaced by the so called “next generation” oncolytic viruses in which the E3B region is

replaced with a therapeutic transgene[37]. This is described mainly under the gene transfer section.

B. HSV

Herpes Simplex virus type 1 is an enveloped double stranded DNA virus. It is a common pathogen in humans, which produces primarily mucosal lesions and can be controlled with the antiviral acyclovir and its derivatives. It is also attractive for virotherapy because as much as 30 kb of its genome can be replaced with artificial genes.

Oncolytic HSV-1 contains mutations that decrease the virulence of the pathogen and increase the neoplastic specificity. Since the most dangerous capability of HSV-1 is the infection of the brain and subsequent temporal lobe encephalitis, it was important to decrease the neurovirulence of the virus. This was done with a mutation in the ICP34.5 and ICP6 loci and insertion of the E.coli LacZ gene.

Thymidine kinase is essential for the replication of the DNA virus, and U_L23-negative mutants of HSV-1 show decreased neurovirulence. Studies using U_L23-negative strain *dl*sptk in hopes of targeting actively mitotic tumor cells, which upregulate their endogenous TK and so may bypass the virus' requirement for U_L2. Experimentation this strain so far has shown strong results in murine and primate models, and completion of a Phase I trial has shown positive results in malignant brain gliomas. A Phase II trial is now commencing comparing it alone or with radiotherapy treatment in malignant brain gliomas[38].

Another HSV-1 variant is the NV1020 virus that contains a deletion in its thymidine kinase gene. As such it relies on human cells for its replication and as thymidine kinase is usually up regulated in neoplastic tissue, the virus responds by more specific growth in those tissues. Murine studies have shown a greater oncolytic power than *dl*sptk, especially in studies using head and neck cancers. An early Phase I trial has concluded that it was safe to administer in metastatic colorectal carcinoma[39].

C. VZV

Vaccinia viruses have also been engineered to selectively lyse tumor cells. The vaccinia virus contains a thymidine kinase gene, much akin to the herpes variety, and so mutants can act in the same manner. Furthermore, varieties of vaccinia have been engineered to include PSA, CEA and GM-CSF genes in the intention of targeting those specific tumors. Results of these studies are still pending.

Oncolysates

This approach uses virus augmented tumor cells (oncolysates) in order to elicit a systemic immune response against a tumor. This approach enhances the antigenicity of the tumor and can evoke an active anti-tumor immune response. Furthermore the viruses can be engineered to produce certain tumor specific antigens and therefore elicit a larger response. Such genes have included those for PSA or CEA. A phase I trial using a vaccine virus encoding for PSA in patients with prostate cancer demonstrated specific T cell responses and was able to inhibit the disease in several patients. Enhancement can improve the response with either the virus expressing IL-2 or GM-CSF or its subsequent administration.

Viral Oncolysis and Chemotherapy

The effect of viral oncolysis can also be enhanced by the addition of chemotherapy. ONYX-015 virus showed greater clinical efficacy when used in combination with chemotherapeutics (5-FU, cisplatin) than as a single agent. Other modified adenoviruses have shown similar effects however, the effect was dependent on dose and sequencing of the agents. A study using a E1A modified adenovirus and gemcitabine demonstrated in mice an increased survival rate than either treatment alone, with almost 60% of treated mice being cured. The author philosophized that the combined effect was due to either a chemosensitizing activity of E1A and/or altered replication kinetics. The mice remained free from disseminated disease, yet did ultimately succumb to chemotherapeutic related hepatic toxicity, which might indicate an increase in toxicity to chemotherapeutics in combined trials[40].

Viral Oncolysis and Radiation Therapy

A very interesting effect is the synergistic effect of viral therapy along in combination with radiotherapy. Amazingly radiation does not impair viral replication and in a number of studies actually increases it. Furthermore, the toxicity is not increased after dual therapy. Lastly in almost all studies of all viruses, dual treatment showed a synergistic anti-tumor effect.

A trial using an adenovirus against prostate cancer followed by radiation resulted in a 6.7 gold greater anti-tumor effect that the predicted additive effect of both therapies[41].

Future directions

Both the history of virus infection in oncology and the current trials highlight the incredible power to which in available with this methods. However the human trials still demonstrate many hurdles that must be overcome before this viral oncotherapy is main stream. Patients have antibodies to the most common viruses. Replication competent viruses possess the possibility of disease and therefore must be applied with caution. The best results so far in this field has come in combination with chemotherapy or radiotherapy.

Oncologic Viruses in Clinical Trials

Parent Virus	Oncolytic agent	Genetic alteration	Cancer targeted	Clinical phase of trial
NDV	None	None	Hematologic	II
Reovirus	None	None	Melanoma	II
			Glioma	II
Adenovirus		E1B		
		E1A		
HSV	G207			I
	NV1020		Colorectal	I/II

Gene Transfer

The newest modality to evolve is the concept involving transfer of a therapeutically acting gene into cancerous cells. The most common vector for the transfer is the replication incompetent adenovirus, however other methods including naked DNA transfer, oligodendromer DNA coatings, electroporation and other virus vectors have been and are being experimented with. The genetic options for transfer include cellular apoptosis genes, antiangiogenesis genes and cellular stasis genes. Gene transfer is a delicate science in that in the design of the insertion gene, care must be taken not to insert the gene into a place that promotes cancer such as a tumor suppressor gene. Furthermore, delivery of the vector to the tissue of interest has to be more precise as unwanted delivery of these genes may preclude potential unwanted consequences in normal functioning tissue.

1. Anti-angiogenic Genes

Due to unregulated localized cellular growth, there comes a period where the perfusion of tissue is inadequate for continued growth. The cells are starved of adequate oxygen and nutrients and are unable to rid themselves of cell metabolic products like carbon dioxide and lactate. It is then imperative for the tumor's continued growth to have a mechanism which will stimulate blood vessel development and permit continued perfusion. Angiogenesis is a tightly regulated mechanism and involves the rapid proliferation of endothelial vascular cells under the control of certain activator and inhibitor growth factors. It is this anti-angiogenesis effect to which exists a mechanism to inhibit and possibly shrink existing tumors.

The first target of anti-angiogenesis was vascular endothelial growth factor (VEGF), found to be amongst the principle promoters of angiogenesis. Its activity has been found to be increased in a number of tumors, especially high grade tumors like glioblastomas. In addition, receptors for VEGF, such as Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2) were found to be overexpressed in relation to normal tissue. Trials blocking the activity of VEGF using anti-sense VEGF cDNA impaired tumor growth either by transfection or via retrovirus transfer.

A number of other physiological proteins have been employed in the trials against tumor angiogenesis. These include interleukin 4 and interferon gamma and have shown improved survival and decreased tumor growth rates. Inevitably neoplastic growth exceeds the existing vasculature and supply of nutrients to permit its growth. For the cells to continue to live and ultimately proliferate further, they must stimulate the body to grow new vessels into their area. The insertion of genes that prevent the growth of vessels essential for tumorous growth and metastases is one method of gene transfer. There are two basic strategies for the control of tumor angiogenesis: 1) deliver molecules with anti-angiogenic activity or 2) produce agents that neutralize the activity of angiogenic factors.

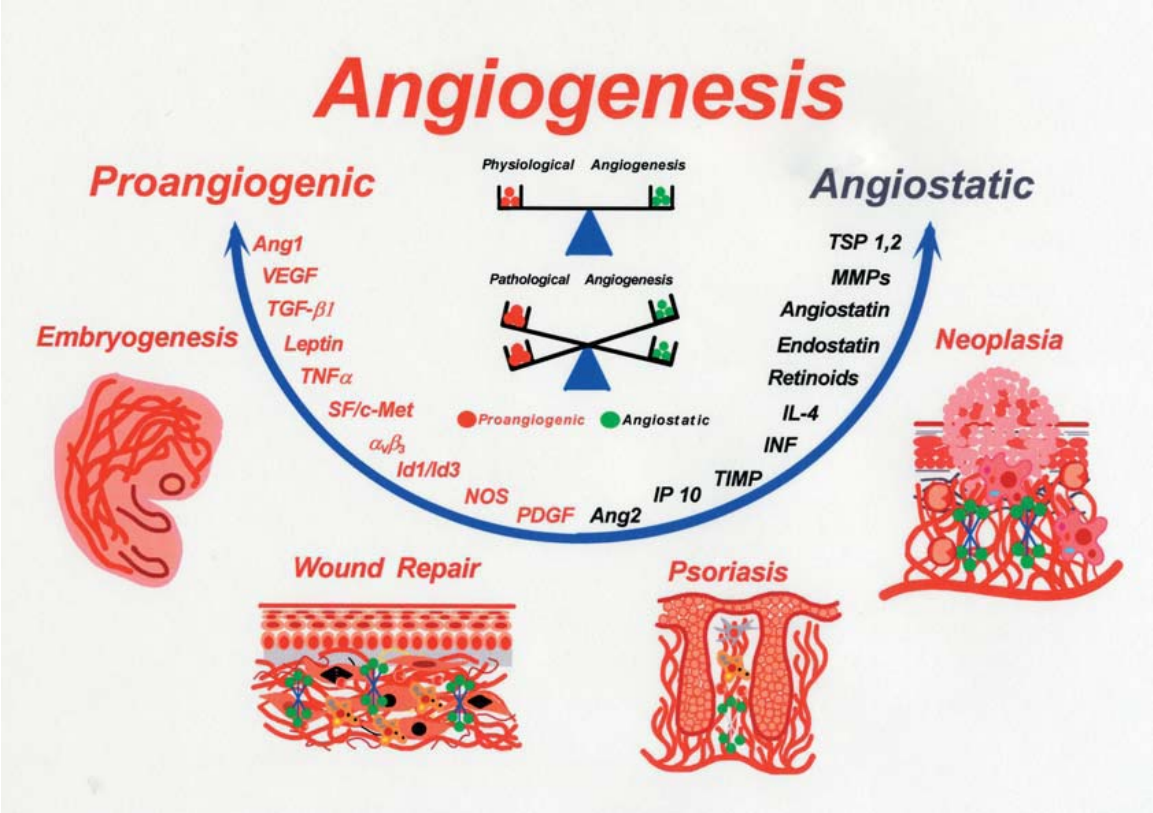

Figure 2. Proangiogenic and Angiostatic factors[42].

The identification of a number of natural inhibitors of angiogenesis has sped up this approach to anti tumor therapy. Principal amongst them are endostatin and angiostatin and are derived from proteolytic cleavage of plasminogen and collagen. Using intratumoral delivery of an adenoviral vector expressing endostatin, one group showed a decreased neoplastic cell proliferation by 57.2% in xenografted hepatocellular carcinoma in mice. After 6-week treatment with this adenovirus expressing endostatin, the growth of treated tumors was inhibited by 46.50%[43]. Another study using angiostatin-like molecule and comparing its efficacy to endostatin showed that angiostatin-like molecule had greater effect on endothelial cell proliferation, migration, and tube formation in vitro[44]. Thrombospondin, an extracellular glycoprotein involved in both angiogenesis and anti-angiogenesis is another target for therapy. The development of an adenovirus expressing the anti-angiogenesis subunit (TSP-1(f)), and administratin to mice with xenografted myelogenous leukemia decreased the microvessel density and cellular proliferation dramatically[45].

The angiogenic factor vascular endothelial growth factor (VEGF) has also been targeted in neoplastic angiogenesis. Using adenoviruses expressing a soluble VEGF receptor, single chain antibodies or ribozymes showed a decrease in lung metastases[46] and inhibited tumor growth in a fibrosarcoma model[47]. Another benefit of these therapies was that they did not require intratumoral delivery, therefore systemic administration or depot delivery was possible.

A number of studies have combined various potential therapies to maximize anti-tumoral effects. Introducing anti-angiogenic genes into oncolytic viruses has improved

the overall efficacy. One study using a E1B deficient adenovirus expressing a gene coding for endostatin showed higher anti-angiogenic effect than with a non-replicating adenovirus and synergistic inhibition of tumor growth[48]. Similar results were demonstrated using a E1B deficient adenovirus encoding soluble VEGF receptor 1[49]. This combination is a promising strategy with a high potential and future clinical trials will be interesting.

2. Targeted Toxins

The continued search for protein surface uniqueness that clearly delineates a cancerous cell from a normal cell is only now starting to identify certain proteins. Antigens such as variants of the IL-13 receptor[50], the urokinase-type plasminogen activator (uPA) receptor Todhunter et al. 2004) and the epidermal growth factor (EGF) receptor[51] are all overexpressed in human gliomas, but are virtually absent in normal brain tissue. The targeted toxin approach used the modified ligands to these receptors and attaches cytotoxic products. The cytotoxic products used have been bacterial toxins, such as the Pseudomonas and Diphtheria exotoxins. The ligand-toxin compound are internalized by the cell, protein synthesis is inhibited which induces cell death. One glioma study in mice using the IL-13 receptor, found that IL-13/cytotoxin combination mediated tumor regression and prolonged survival of animals by 164% compared with control[52].

3. Suicide Gene Therapy

Suicide gene therapy or gene directed enzyme prodrug therapy is where an enzyme is incorporated into the cell in question, and modifies the drug applied afterwards to make it cytotoxic. The enzymes are usually nonhuman, although they must resemble human enzymes inorder to be incoporated into replicated DNA. Furthermore the prodrug must be selectively activated by the enzyme and be efficient at killing the cell. Both the enzyme and prodrug must have high distributive and infective properties because transduction of cells is not an efficient mechanism, and successful treatment relies on the transfer between cells, the so called “bystander effect.” A total of 42 prodrugs explored for use in suicide gene therapy with 12 different enzymes are available.

A. HSV-1 Thymidine Kinase

Thymidine Kinase is a cellular enzyme that phosphorylates deoxythymidine as an early step in the incorporation of deoxythymidine into replicated DNA. The enzyme exists in two isoforms TK1 present in the cytoplasm of dividing cells and is cell cycle dependent and TK2, which exists in the mitochondria and is cell cycle independent. Viral TK1 has a much larger spectrum of activity than human. It is able to phosphorylate other deoxythymidine resembling substances. This is the specific effect of the antiviral medications acyclovir and ganciclovir. Both are activated from a non toxic prodrug to a toxic compound after activation by Herpes Simplex virus type 1 thymidine kinase in affected cells. Numerous studies have shown an antitumor effect after as little as 10% of tumor cells was infected. This is the so called “bystander effect”, whereby surrounding cells are effected typically via transfer of ganciclovir through gap junctions.

Numerous vectors have been utilized for the transfer of HSV-1 TK1 into neoplastic cells: replication deficient retroviruses and adenoviruses, replication competent adeno-associated virus and HSV. All trials showed a cytotoxic T lymphocyte mediated response and regression of tumor. In addition treatment with HSV-1 TK/ganciclovir rendered all cells more susceptible to the adjunctive treatment of chemotherapy and radiation.

The earliest trials have been with brain gliomas. A Phase III trial using HSV-1 TK followed by ganciclovir showed no benefit against standardized treatment or surgery followed by radiotherapy in 248 patients[53]. The trial did highlight the difficulty in intratumoral injection, especially in the brain and low therapeutic concentration achieved across the blood brain barrier of ganciclovir.

Numerous mutations are being used to try to increase the Km value of TK1. Also other treatment options are being added to attempt to increase the cancer cell cytotoxicity, including other cytostatics like 5-fluorouracil and Tomudex, proteases trypsin and collagenase, radiotherapy and GM-CSF to increase the immune response. GM-CSF expression in HSV-1 TK/ganciclovir exposed tumors has shown a very good response in murine models. While tumor reappearance has been almost universal in murine tumor models after treatment with HSV-1 TK/ganciclovir, the transfer of GM-CSF demonstrated an 80% better cure rate than without, demonstrating the ongoing role of the immune response.

Use of esterified ganciclovir elaidic acid has also increase the effectivity of the HSV-1 TK/ganciclovir combination. Since it was much more lipophilic the duration of action was much longer and also the potency is higher[54]. Alternatively other antiherpetic medications with similar therapeutic effects have included penciclovir, acyclovir and valaciclovir. In addition, E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) a potent antiherpes agent enhances the GCV-induced killing of HSV-Tk transduced cells. It is a better substrate for use with varicella zoster virus (VZV) thymidine kinase if used independently, but demonstrates a poor bystander effect. Similar action is observed in HSV-1 TK mutants with the potent anti-HIV medication AZT.

Malignancy	Antiviral agent	Phase
Hematological		I, II
Brain gliomas	Ganciclovir	I
Melanoma	Ganciclovir	I
Brain gliomas	Ganciclovir sodium	I

B. Cytosine Deaminase

The next most commonly used in cytosine deaminase (CD). This enzyme is present only in fungi and prokaryotes, but not in multicellular eukaryotes and catalyzes the conversion of cytosine to uracil. Cytosine deaminase is a crucial enzyme in the pyrimidine salvage pathway. The studies with CD have focused almost exclusively on one prodrug: 5-fluorocytosine. Only via activation with CD is 5-fluorocytosine converted to its active form, 5-fluorouracil, and can lyse infected cells. Early trials with E. coli derived CD on human glioblastoma cells, highlighted the high potential of this therapy along with the hurdles that must be overcome. There was very slow uptake of

the naked DNA by passive diffusion and rapid efflux. 5-fluorouracil has proven to be the most effective chemotherapeutic drug for colon cancer being converted by cellular enzymes to the ribosyl monophosphate 5-FdUMP, which is an irreversible inhibitor of thymidylate synthetase. Early in vitro studies showed a 200-fold increase in neoplastic cell sensitivity to 5-FC in cells expressing CD than the nonexpressing cell lines **(**Cytosine deaminase gene as a potential tool for the genetic therapy of colorectal cancer.) At least 90% of the cells are killed within 7 days. In contrast to GCV, the bystander effects of CD/5-FC therapy do not depend on gap junctional intercellular communication (GJIC), and very large effects are seen with both communication-competent and -incompetent cells, mediated by the diffusion of 5-FU. It has been suggested that CD/5-FU therapy in solid tumor models can generate complete cures if only 4% of the tumor cell mass express the enzyme[55]. Breast carcinoma cells transfected with *E. coli* CD were sensitized 1000 fold to 5-FC in culture, with only 10% of the infected cells needed to induce complete cytotoxicity.

Further studies have incorporated CD with other suicide genes in an attempt to upregulate the cell specific killing. The addition of E. coil uracil phosphoribosyltransferase (UPRT), which is a pyrimidine salvage enzyme and directly converts 5-fluorouracil (5-FU) to 5-fluorouridine monophosphate at the first step of its activating pathway, was shown to improve the antitumoral effect of the CD/5-FC system. This combination demonstrated an additive effect and furthermore increased the cellular sensitivity to chemotherapeutic treatment. Another successful trial used combined suicide gene therapy for human colon cancer cells using adenovirus-mediated transfer of escherichia coli cytosine deaminase gene and Escherichia coli uracil phosphoribosyltransferase gene with 5-fluorocytosine. Colorectal trials have incorporated the CD gene along with a carcinoembryonic antigen (CEA) promoter. CEA has been proven to be expressed in 50% of colorectal cancers. Human telomerase reverse transcriptase (hTERT), the catalytic subunit of the telomerase, is transcriptionally upregulated in more than 90% of tumor cells. Via a plasmid transfer of CD attached to a hTERT promoter, the upregulation of hTERT could increase genetic expression of CD in a cancer specific manner. It was shown that the expression of the CD gene increased the sensitivity of cells with the hTERT promoter and CD over 800-fold, versus only 6 fold if only CD was used after 5-fluorocytosine treatment.

Another study has utilized the combination adenoviral introduction of CD along with TK to increase suicide gene killing. Furthermore the expression of both genes is under the control of VEGF, known to be upregulated in tumors.

Early gene transfer trials suffered from gene silencing. That is if they were effectively introduced by the appropriate vector into the cell, the gene was not expressed in a significant proportion for a therapeutic effect. Consequently, the incorporation of either a cell specific promoter or enhancer has increased the expression of the gene in question and also made the selectivity higher.

When 5-FC/CD and HSV-Tk/GCV therapies were compared in a variety of in vivo models, both appeared of similar efficacy in hepatocellular carcinoma[56], but CD/5-FC was clearly superior in EBV-associated lymphomas[57], renal cell carcinoma[58], and colorectal carcinoma[59]. This is probably attributed to its superior bystander effect. Combination studies of 5-FC with radiotherapy in CD-transfected tumors also shown sensitization of subcutaneous xenografts of squamous cell carcinoma[60],

cholangiocarcinoma and colon carcinoma[61], using a dose of 800 mg/kg/day of 5-FC and from 10–50 Gy of tumor irradiation.

C. Purine Nucleoside Phosphorylase

PNP is an enzyme in the purine salvage pathway that metabolizes inosine and guanosine to hypoxanthine. The E.coli variant converts nontoxic purine nucleoside analogs into toxic adenine analogs to block both mRNA and protein synthesis. The most commonly used prodrug for GDEPT is 6-methylpurine deoxyriboside (MEP) which is converted to a highly diffusible metabolite with excellent bystander effects independent of cell to cell contact. Human ovarian tumors transfected with *E. coli* PNP controlled by an SV40 promoter and implanted IP were shown to express PNP in only 0.1% of the cells after 5 days, yet treatment of these with MEP resulted in an average 49% reduction in tumor size and 30% increase in life span compared with control tumors. Also found was an in vivo sensitization of ovarian tumors to chemotherapy by expression of E. coli PNP[62]. A comparison of MEP/PNP and GCV/HSV-Tk therapy in a PC-3 human androgen-independent prostate cancer cell line showed that MEP/PNP caused more rapid cell killing at a 5–10-fold lower input of virus[63]. Against the same cells as sc tumors in nude mice, both systems showed comparable activity, holding tumor growth to about 75% of that of controls after 52 days, and providing about 20% of long-term survivors[64].

Fludarabine has also shown good effect after pretreatment with PNP. Studies in hepatoma cells and glioma lines proved more effective than treatments with TK.

D. CYP Enzymes

NADH cyto-chrome P450 (CYP) enzymes are detoxification enzymes expressed most highly in liver cells, however are also more highly active in tumor cells.

E. Carboxypeptidase G2

Carboxypeptidase G2 (CPG2) is a bacterial enzyme that removes glutamic acid moieties from folic acid thereby inhibiting cell growth. It can be combined with the prodrug 4-benoyl-L-glutamic acid (CMDA) with the release of a mustard gas drug. The mustard alkylating agent released is not cell cycle dependent and therefore has the beneficial effect of killing proliferating and non-proliferating cells. One study in glioma cells, showed a 70% cell killing with CPG2 and CMDA after the cells had become resistant to chemotherapy and not killed by HSV-1 TK/GCV treatment[65]. Trials have continued with the used of the more potent hydroxy- and amino-aniline mustards that are up to 70 fold better killers.

Alternative DNA transfer Methods

Oligonucleotides are short single-stranded segments of DNA that upon cellular internalization can selectively inhibit the expression of a single protein. They can form either antisense complexes with mRNA or antigen triplexes with DNA, and thereby inhibit transcription or translation. Oligonucleotides such as MG98 and ISIS 5132 are designed to inhibit the biosynthesis of DNA methyltransferase and c-raf kinase.

Ribozymes are RNA molecules that are capable of sequence specific cleaving of mRNA molecules. They bind to the target molecules, form a duplex and hydrolyze the mRNA molecule. RNA being very unstable and susceptible to RNase degradation makes these molecules particularly unstable. Ribozymes have been used for gene knockout therapy by targeting overexpressing oncogenes such as the human epidermal growth factor receptor Type 2 gene, that is implicated in breast cancer and HPV infection.

DNAzymes are similar to ribozymes but have the advantage of greater stability to work with and within the cell. A DNAzyme has been synthesized against VEGF receptor 2 and its effect was confirmed by blocking angiogenesis upon intratumoral injection in mice[66].

Aptamers are small single-stranded or double-stranded nucleic acid segments that can directly interact with proteins. They are less immunogenic and more specific and stabile that antibodies. Interacting with proteins involved in the functions or transcription and translation is their therapeutic idea. One trial involves the uses of a anti-VEGF aptamer for macular degeneration. It could also theoretically be used for anti-angiogenesis in cancer therapy.

Small Interfering RNAs (SiRNAs) can be used for the downregulation of disease-causing gene through RNA interference. They are short double stranded RNA segments that are complementary to the mRNA sequence, and therefore block the translational activity of that mRNA. They are much more specific and stabile to ribonucleases than oligonucleotides.

Limitations

The most important hurdle to overcome in the evolution of gene transfer is that of the host cell-mediated immune responses against the transduced cells. Cytotoxic T lymphocyte responses against viral proteins or the transgene product can result in either destruction of the transduced cells or cessation of the transgene expression[67]. Viral vectors manufactured to express as little viral gene products as possible (gutted viral vectors) may be more advantageous as would pretreatment with certain immunosuppressants.

Conclusion

Although the possibilities for gene therapy in cancer treatment have come a long way, with tissue and animal models showing good results, the clinical results to date are still disappointing. More needs to be understood of the immune response to foreign pathogens, and how we can adjust its effect. Whether it upregulate a response using immunotherapy or downregulate a response with the introduction of a virus from

oncolysis or gene transfer. We need to further understand the "bystander effect" and how far different drugs diffuse through tissues.

What is exciting is the results been seen especially in the combination treatments. While the basic modalities of cancer treatment, surgery, radiotherapy and chemotherapy, will remain for the short time the prime treatments, it is not unconceivable to guess that gene therapy will be an accepted adjuvant therapy in the near future.

References

[1] http://www.euro.who.int/document/ehfa5-e.pdf. 2007.

[2] http://www.cancer.org/downloads/STT/Caff2006PWSecured.pdf. 2007.

[3] Simons JW, Sacks N: Granulocyte-macrophage colony-stimulating factor-transduced allogeneic cancer cellular immunotherapy: the GVAX vaccine for prostate cancer. Urol Oncol 24:419-424, 2006.

[4] Leong SP, Enders-Zohr P, Zhou YM, Stuntebeck S, Habib FA, Allen RE, Jr., Sagebiel RW, Glassberg AB, Lowenberg DW, Hayes FA: Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) and autologous melanoma vaccine mediate tumor regression in patients with metastatic melanoma. J Immunother 22:166-174, 1999.

[5] Berzofsky JA, Terabe M, Oh S, Belyakov IM, Ahlers JD, Janik JE, Morris JC: Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J Clin Invest 113:1515-1525, 2004.

[6]Minami T, Matsueda S, Takedatsu H, Tanaka M, Noguchi M, Uemura H, Itoh K, Harada M: Identification of SART3-derived peptides having the potential to induce cancer-reactive cytotoxic T lymphocytes from prostate cancer patients with HLA-A3 supertype alleles. Cancer Immunol Immunother 56:689-698, 2007.

[7] Tagawa ST, Cheung E, Banta W, Gee C, Weber JS: Survival analysis after resection of metastatic disease followed by peptide vaccines in patients with Stage IV melanoma. Cancer 106:1353-1357, 2006.

[8] Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE: Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 4:321-327, 1998.

[9]. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Sznol M, Schwarz SL, Spiess PJ, Wunderlich JR, Seipp CA, Einhorn JH, Rogers-Freezer L, White DE: Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. J Immunol 163:1690-1695, 1999.

[10] Roos AK, Pavlenko M, Charo J, Egevad L, Pisa P: Induction of PSA-specific CTLs and anti-tumor immunity by a genetic prostate cancer vaccine. Prostate 62:217-223, 2005

[11] Gene Ther 2006.Hurwitz, Arthur A., Tina F.-Y. Yu, Dana R. Leach, James P. Allison. CTLA-4 blockade synergizes with tumor-derived granulocyte– macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. Proc Natl Acad Sci U S A. 1998 August 18; 95(17): 10067–10071.

[12] Hodi, F. Stephen, Martin C. Mihm, Robert J. Soiffer, Frank G. Haluska, Marcus Butler, Michael V. Seiden, Thomas Davis, Rochele Henry-Spires, Suzanne MacRae, Ann Willman, Robert Padera, Michael T. Jaklitsch, Sridhar Shankar, Teresa C. Chen, Alan Korman, James P. Allison, and Glenn Dranoff. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci U S A. 2003 April 15; 100(8): 4712–4717.

[13] Jun Shimizu*, Sayuri Yamazaki† and Shimon Sakaguchi2,*,† Induction of Tumor Immunity by Removing CD25+CD4+ T Cells: A Common Basis Between Tumor Immunity and Autoimmunity, J Immunol. 1999 Nov 15;163(10):5211-8.

[14] Sutmuller, Roger P.M., Leonie M. van Duivenvoorde, Andrea van Elsas, Ton N.M. Schumacher, Manon E. Wildenberg, James P. Allison, Rene E.M. Toes, Rienk Offringa, and Cornelis J.M. Melief. Synergism of Cytotoxic T Lymphocyte–associated Antigen 4 Blockade and Depletion of CD25+ Regulatory T Cells in Antitumor Therapy Reveals Alternative Pathways for Suppression of Autoreactive Cytotoxic T Lymphocyte Responses. The Journal of Experimental Medicine, Volume 194, Number 6, September 17, 2001 823-832.

15 Ferlazzo G, Wesa A, Wei WZ, Galy A. Dendritic cells generated either from CD34+ progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8+ T cells. J Immunol. 1999 Oct 1;163(7):3597-604.

[16] Merad, Miriam, Tomoharu Sugie, Edgar G. Engleman, and Lawrence Fong. In vivo manipulation of dendritic cells to induce therapeutic immunity. Blood, 1 March 2002, Vol. 99, No. 5, pp. 1676-1682.

17 Heiser, Axel., et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. The Journal of Clinical Investigation. Feb. 2002. 109:409-417.

18 Hung, Kenneth, Robert Hayashi, Anne Lafond-Walker,‡ Charles Lowenstein, Drew Pardoll, and Hyam Levitsky. The Central Role of CD4+ T Cells in the Antitumor Immune Response. J. Exp. Med., Volume 188, Number 12, December 21, 1998 2357-2368.

[19] Khan JA, Yaqin S: Successful immunological treatment of gallbladder cancer in India--case report. J Zhejiang Univ Sci B 7:719-724, 2006.

[20]Tan G, Wang ZY, Wang XG, Cheng L, Yin S: [Immunotherapeutic effects of beta-elemene combined with interleukin-23 gene-modified dendritic cells on murine pancreatic carcinoma]. Ai Zheng 25:1082-1086, 2006.

[21]Met O, Wang M, Pedersen AE, Nissen MH, Buus S, Claesson MH: The effect of a therapeutic dendritic cell-based cancer vaccination depends on the blockage of CTLA-4 signaling. Cancer Lett 231:247-256, 2006.

[22]Chen Z, Xia D, Bi X, Saxena A, Sidhu N, El Gayed A, Xiang J: Combined radiation therapy and dendritic cell vaccine for treating solid tumors with liver micro-metastasis. J Gene Med 7:506-517, 2005.

[23] Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, Taquet S, Coquery S, Wittkowski KM, Bhardwaj N, Pineiro L, Steinman R, Fay J: Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 61:6451-6458, 2001.

[24]Fong TC, Sauter SL, Ibanez CE, Sheridan PL, Jolly DJ: The use and development of retroviral vectors to deliver cytokine genes for cancer therapy. Crit Rev Ther Drug Carrier Syst 17:1-60, 2000.

[25]Chen Z, Xia D, Bi X, Saxena A, Sidhu N, El Gayed A, Xiang J: Combined radiation therapy and dendritic cell vaccine for treating solid tumors with liver micro-metastasis. J Gene Med 7:506-517, 2005.

[26]Trudel S, Trachtenberg J, Toi A, Sweet J, Li ZH, Jewett M, Tshilias J, Zhuang LH, Hitt M, Wan Y, Gauldie J, Graham FL, Dancey J, Stewart AK: A phase I trial of adenovector-mediated delivery of interleukin-2 (AdIL-2) in high-risk localized prostate cancer. Cancer Gene Ther 10:755-763, 2003.

[27]Elankumaran S, Rockemann D, Samal SK: Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. J Virol 80:7522-7534, 2006.

[28]Figova K, Hrabeta J, Eckschlager T: Reovirus - possible therapy of cancer. Neoplasma 53:457-462, 2006.

[29]Kaplan JM: Adenovirus-based cancer gene therapy. Curr Gene Ther 5:595-605, 2005.

[30]Rothmann T, Hengstermann A, Whitaker NJ, Scheffner M, zur HH: Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. J Virol 72:9470-9478, 1998.

[31]Heise C, Hermiston T, Johnson L, Brooks G, Sampson-Johannes A, Williams A, Hawkins L, Kirn D: An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat Med 6:1134-1139, 2000.

[32]Edelman J, Edelman J, Nemunaitis J: Adenoviral p53 gene therapy in squamous cell cancer of the head and neck region. Curr Opin Mol Ther 5:611-617, 2003.

[33]Katze MG: Interferon, PKR, virology, and genomics: what is past and what is next in the new millennium? J Interferon Cytokine Res 22:283-286, 2002.

[34]Cascallo M, Capella G, Mazo A, Alemany R: Ras-dependent oncolysis with an adenovirus VAI mutant. Cancer Res 63:5544-5550, 2003.

[35]Cascallo M, Gros A, Bayo N, Serrano T, Capella G, Alemany R: Deletion of VAI and VAII RNA genes in the design of oncolytic adenoviruses. Hum Gene Ther 17:929-940, 2006.

[36]Pilder S, Moore M, Logan J, Shenk T: The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol Cell Biol 6:470-476, 1986.

[37]Zhan J, Gao Y, Wang W, Shen A, Aspelund A, Young M, Laquerre S, Post L, Shen Y: Tumor-specific intravenous gene delivery using oncolytic adenoviruses. Cancer Gene Ther 12:19-25, 2005.

[38]Todo T, Martuza RL, Dallman MJ, Rabkin SD: In situ expression of soluble B7-1 in the context of oncolytic herpes simplex virus induces potent antitumor immunity. Cancer Res 61:153-161, 2001.

[39]Kemeny N, Brown K, Covey A, Kim T, Bhargava A, Brody L, Guilfoyle B, Haag NP, Karrasch M, Glasschroeder B, Knoll A, Getrajdman G, Kowal KJ, Jarnagin WR, Fong Y: Phase I, Open-Label, Dose-Escalating Study of a Genetically Engineered Herpes Simplex Virus, NV1020, in Subjects with Metastatic Colorectal Carcinoma to the Liver. Hum Gene Ther 2006.

[40]Raki M, Kanerva A, Ristimaki A, Desmond RA, Chen DT, Ranki T, Sarkioja M, Kangasniemi L, Hemminki A: Combination of gemcitabine and Ad5/3-Delta24, a

tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer. Gene Ther 12:1198-1205, 2005.

[41]Quigg M, Mairs RJ, Brown SM, Harland J, Dunn P, Rampling R, Livingstone A, Wilson L, Boyd M: Assessment in vitro of a novel therapeutic strategy for glioma, combining herpes simplex virus HSV1716-mediated oncolysis with gene transfer and targeted radiotherapy. Med Chem 1:423-429, 2005.

[42]Polverini PJ: Angiogenesis in health and disease: insights into basic mechanisms and therapeutic opportunities. J Dent Educ 66:962-975, 2002.

[43]Li GC, Nie MM, Yang JM, Su CQ, Sun LC, Qian YZ, Sham J, Fang GE, Wu MC, Qian QJ: [Treatment of hepatocellular carcinoma with a novel gene-viral therapeutic system CNHK300-murine endostatin]. Zhonghua Yi Xue Za Zhi 84:943-948, 2004.

[44]Schmitz V, Wang L, Barajas M, Gomar C, Prieto J, Qian C: Treatment of colorectal and hepatocellular carcinomas by adenoviral mediated gene transfer of endostatin and angiostatin-like molecule in mice. Gut 53:561-567, 2004.

[45]Liu P, Wang Y, Li YH, Yang C, Zhou YL, Li B, Lu SH, Yang RC, Cai YL, Tobelem G, Caen J, Han ZC: Adenovirus-mediated gene therapy with an antiangiogenic fragment of thrombospondin-1 inhibits human leukemia xenograft growth in nude mice. Leuk Res 27:701-708, 2003.

[46] Takei Y, Mizukami H, Saga Y, Yoshimura I, Hasumi Y, Takayama T, Kohno T, Matsushita T, Okada T, Kume A, Suzuki M, Ozawa K: Suppression of ovarian cancer by muscle-mediated expression of soluble VEGFR-1/Flt-1 using adeno-associated virus serotype 1-derived vector. Int J Cancer 120:278-284, 2007.

[47]Afanasieva TA, Wittmer M, Vitaliti A, Ajmo M, Neri D, Klemenz R: Single-chain antibody and its derivatives directed against vascular endothelial growth factor: application for antiangiogenic gene therapy. Gene Ther 10:1850-1859, 2003.

[48]Li G, Sham J, Yang J, Su C, Xue H, Chua D, Sun L, Zhang Q, Cui Z, Wu M, Qian Q: Potent antitumor efficacy of an E1B 55kDa-deficient adenovirus carrying murine endostatin in hepatocellular carcinoma. Int J Cancer 113:640-648, 2005.

[49] Zhang Z, Zou W, Wang J, Gu J, Dang Y, Li B, Zhao L, Qian C, Qian Q, Liu X: Suppression of tumor growth by oncolytic adenovirus-mediated delivery of an antiangiogenic gene, soluble Flt-1. Mol Ther 11:553-562, 2005.

[50]Debinski W, Slagle B, Gibo DM, Powers SK, Gillespie GY: Expression of a restrictive receptor for interleukin 13 is associated with glial transformation. J Neurooncol 48:103-111, 2000.

[51]Liu TF, Hall PD, Cohen KA, Willingham MC, Cai J, Thorburn A, Frankel AE: Interstitial diphtheria toxin-epidermal growth factor fusion protein therapy produces regressions of subcutaneous human glioblastoma multiforme tumors in athymic nude mice. Clin Cancer Res 11:329-334, 2005.

[52]Kawakami K, Kioi M, Liu Q, Kawakami M, Puri RK: Evidence that IL-13R alpha2 chain in human glioma cells is responsible for the antitumor activity mediated by receptor-directed cytotoxin therapy. J Immunother 28:193-202, 2005.

[53]Rainov NG: A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. Hum Gene Ther 11:2389-2401, 2000.

[54]Balzarini J, Degreve B, Andrei G, Neyts J, Sandvold M, Myhren F, De Clercq E: Superior cytostatic activity of the ganciclovir elaidic acid ester due to the prolonged intracellular retention of ganciclovir anabolites in herpes simplex virus type 1 thymidine kinase gene-transfected tumor cells. Gene Ther 5:419-426, 1998.

[55] Huber BE, Austin EA, Richards CA, Davis ST, Good SS: Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc Natl Acad Sci U S A 91:8302-8306, 1994.

[56]Kuriyama S, Nakatani T, Masui K, Sakamoto T, Tominaga K, Yoshikawa M, Fukui H, Ikenaka K, Tsujii T: Bystander effect caused by suicide gene expression indicates the feasibility of gene therapy for hepatocellular carcinoma. Hepatology 22:1838-1846, 1995.

[57]Rogers RP, Ge JQ, Holley-Guthrie E, Hoganson DK, Comstock KE, Olsen JC, Kenney S: Killing Epstein-Barr virus-positive B lymphocytes by gene therapy: comparing the efficacy of cytosine deaminase and herpes simplex virus thymidine kinase. Hum Gene Ther 7:2235-2245, 1996.

[58]Shirakawa T, Gardner TA, Ko SC, Bander N, Woo S, Gotoh A, Kamidono S, Chung LW, Kao C: Cytotoxicity of adenoviral-mediated cytosine deaminase plus 5-fluorocytosine gene therapy is superior to thymidine kinase plus acyclovir in a human renal cell carcinoma model. J Urol 162:949-954, 1999.

[59]Trinh QT, Austin EA, Murray DM, Knick VC, Huber BE: Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. Cancer Res 55:4808-4812, 1995.

[60]Hanna NN, Mauceri HJ, Wayne JD, Hallahan DE, Kufe DW, Weichselbaum RR: Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. Cancer Res 57:4205-4209, 1997.

[61]Gabel M, Kim JH, Kolozsvary A, Khil M, Freytag S: Selective in vivo radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells transduced with the E. coli cytosine deaminase (CD) gene. Int J Radiat Oncol Biol Phys 41:883-887, 1998.

[62] Gadi VK, Alexander SD, Kudlow JE, Allan P, Parker WB, Sorscher EJ: In vivo sensitization of ovarian tumors to chemotherapy by expression of E. coli purine nucleoside phosphorylase in a small fraction of cells. Gene Ther 7:1738-1743, 2000.

[63]Lockett LJ, Molloy PL, Russell PJ, Both GW: Relative efficiency of tumor cell killing in vitro by two enzyme-prodrug systems delivered by identical adenovirus vectors. Clin Cancer Res 3:2075-2080, 1997.

[64]Martiniello-Wilks R, Garcia-Aragon J, Daja MM, Russell P, Both GW, Molloy PL, Lockett LJ, Russell PJ: In vivo gene therapy for prostate cancer: preclinical evaluation of two different enzyme-directed prodrug therapy systems delivered by identical adenovirus vectors. Hum Gene Ther 9:1617-1626, 1998.

[65] Cowen RL, Williams JC, Emery S, Blakey D, Darling JL, Lowenstein PR, Castro MG: Adenovirus vector-mediated delivery of the prodrug-converting enzyme carboxypeptidase G2 in a secreted or GPI-anchored form: High-level expression of this active conditional cytotoxic enzyme at the plasma membrane. Cancer Gene Ther 9:897-907, 2002.

[66]Zhang L, Gasper WJ, Stass SA, Ioffe OB, Davis MA, Mixson AJ: Angiogenic inhibition mediated by a DNAzyme that targets vascular endothelial growth factor receptor 2. Cancer Res 62:5463-5469, 2002.

[67] Kaplan JM: Adenovirus-based cancer gene therapy. Curr Gene Ther 5:595-605, 2005.