Gene Therapy of Cancer

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Cancer is a disease of the genes

Cancer is thought to arise when a cell accumulates a sufficient number of defects in critical genes. These defective genes result in the production of dysregulated growth stimulatory proteins ("dominant oncogenes") or lack of production of key growth limiting proteins ("tumor suppressor genes"). The net effect of this is a cell which, while able to divide, fails to respond to both internal and external cues which control that growth.

Some of these abnormal genes may be inherited, such as mutant p53 in the Li-Fraumeni syndrome[1, 2] and mutant Rb in familial retinoblastoma[3-5], but in the development of the common forms of cancer, most appear to be acquired during the life of the individual. The causes of most of these "somatic" abnormalities is not clear, but the single most common cause of cancer death in the United States is lung cancer, and it is estimated that 80-85% of the cases are caused by exposure to tobacco smoke[6-9], which is a potent mixture of known genotoxins.

Fighting genes with genes

Many standard cancer chemotherapeutic agents are directly genotoxic and others are antimetabolites. There is no intrinsic specificity to these agents to allow them to selectively kill cancer cells and not normal ones, other than the hope that cancer cells are somewhat defective in various salvage and repair pathways. Thus these agents have a much smaller difference between effective and toxic doses ("therapeutic window") than do agents with intrinsic specificity, such as penicillin.

In spite of cancer's polygenic nature, it has been found that correction of one of the genetic defects in cancer cells *in vitro* can lead to the reversal of tumorigenicity[10, 11]. If this could be done in human tumors *in vivo*, it might constitute an effective cancer therapy. For oncogenes, the situation is similar to genetic correction of inherited single gene defects, such as adenosine deaminase deficiency, or cystic fibrosis. Absent or defective tumor suppressor genes this function could be replaced by the insertion of the wild-type gene, and for dominant oncogenes, expression can be reduced by means which we will discuss below. In addition to genes which act directly via oncogenes, there are a variety of other genes which, when introduced into host cells or tumor cells, can "unmask" tumor antigens and result in an effective anti-tumor immune response, induce specific drug sensitivity in tumor cells, or protect the host from the harmful effects of chemotherapy. These approaches will be briefly reviewed below.

"Gene Therapy" of Cancer

Gene therapy of cancer can be defined as the use of DNA or related derivatives as anticancer agents. This DNA can be delivered to tumor cells

or normal cells, by infectious or non-infectious methods, and alter a variety of normal or abnormal cellular processes, or even introduce new pathways where none existed previously. There are many fundamental questions that need answers before a practical cancer gene therapeutic protocol can be achieved.

Fundamental Questions:

- which vector?
- how is that vector delivered?
- what target tissue?
- what gene?
- what is a useful model system?
- what is the mechanism of tumor inhibition?

Vectors

By "vector" I mean the form that the genetic information takes. This can be a recombinant virus or plasmid, entirely synthetic, etc.

Retroviruses

This is the most often used vector system. Retroviruses are positive strand RNA viruses which must be reverse transcribed into DNA and integrated into the genome for gene expression to occur. Once integrated into the genomic DNA, the provirus has the structure shown in figure 1 with the coding region flanked by direct repeats containing the viral promoters. Viral RNA reverse transcription and integration occur as shown in figure 2.

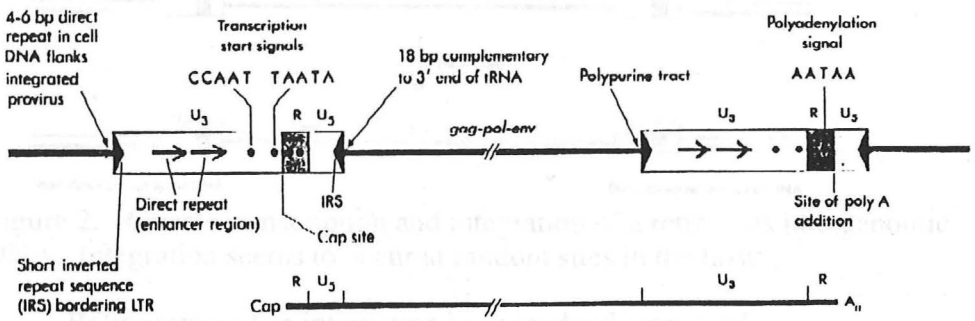


Figure 1. The structure of an integrated retrovirus (provirus). Gag, pol and env are viral genes essential for replication of the virus.

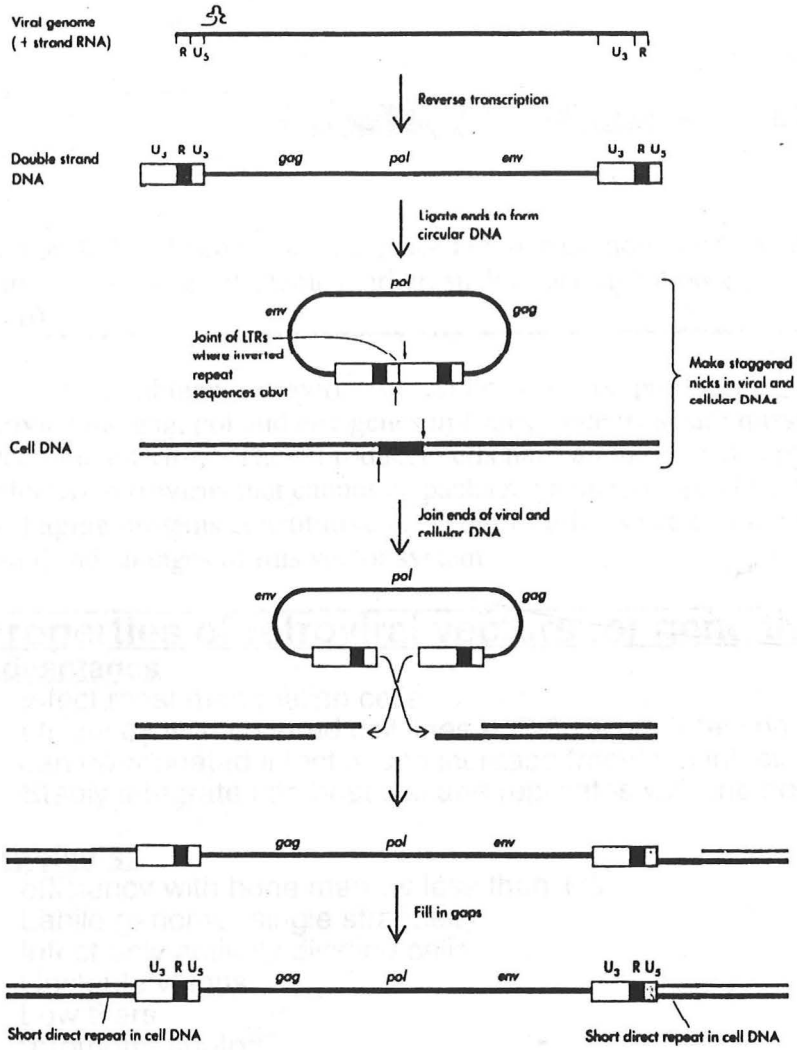


Figure 2. Reverse transcription and integration of a retrovirus into genomic DNA. Integration seems to occur at random sites in the host.

Retroviruses after integration have tandemly repeated promoter/enhancer regions called "Long Terminal Repeats" or LTRs flanking the viral genome. The retroviral genes gag, pol, and env can be replaced by recombinant genes either driven off the retroviral LTR promoter or an internal promoter (e.g. CMV). Deletion of essential viral genes makes replication dependent on the presence of a "helper virus".

Figure 3. Viral genes can be replaced by one or more recombinant genes with or without a selectable marker, such as neomycin phosphotransferase (neo).

Recombinant retroviruses must be grown on producer cells which provide the gag, pol and env genes in *trans*, since these are missing from the recombinant virus. These producer cells have an integrated copy of a defective retrovirus that cannot be packaged alone, but produce the required packaging proteins constitutively. Table 1 outlines some of the advantages and disadvantages of this vector system.

Properties of retroviral vectors for gene therapy:

Advantages

- Infect most mammalian cells
- efficiency with cultured cell lines ~30-60% per infection
- can do repeated infections to increase fractional infection.
- Stably integrate into host cell and replicates with the host genome.

Problems:

- efficiency with bone marrow less than 1%
- Labile genome (single stranded)
- Infect only actively dividing cells
- Unstable virions
- Low titers
- "promoter shutoff"

Table 1.

Adenoviruses

Adenoviral vectors are being increasingly utilized for gene therapeutic maneuvers. Adenoviruses are linear, double stranded DNA viruses, approximately 35 kilobases long, and encode at least 50 polypeptides.

Lytic infection proceeds in two phases: the early phase which preceeds the onset of viral replication, and the late phase that depends on

viral DNA synthesis. Late gene expression is dependent on early gene expression, particularly the E1a and E1b genes.

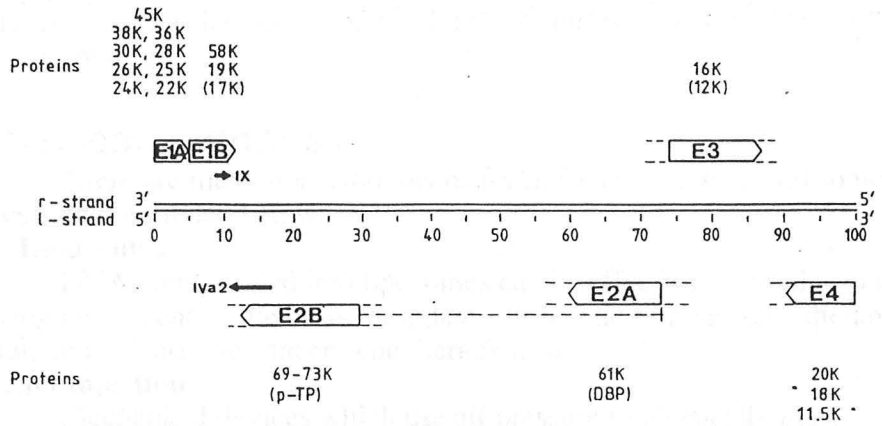


Figure 4. Adenovirus early genes.

Recombinant adenoviruses typically replace the early region gene E1a with your gene of choice. This is done by *in vivo* recombination between a shuttle plasmid and a viral genome clone. This renders the recombinant virus unable to express its late proteins after infection, and requires that recombinant virus be grown on a cell line which constitutively expresses the E1a gene (293 cells).

Properties of adenoviral vectors

Advantages

- can be grown to titers 100-10000 fold higher than retroviruses.
- infect cells in stationary phase
- higher single infection efficiency.

Problems

- low efficiency of integration: most cells express transiently
- current vectors have size limitation to inserts
- expression of E3 protein may inhibit MHC class I expression in infected cells

Table 2.

Other viruses

Herpes virus, vaccinia virus, adeno-associated virus and others have been tried. None of these are as developed as retroviruses or adenoviruses.

Recently it has been shown that DNA can be carried into cells by putting polylysine tracts onto inactivated adenovirus capsids. This approach circumvents the size limitation for the DNA, but is 10-100 fold less efficient than viral infection.

Physical methods

There are many non-viral means for introducing DNA, and some of these are mentioned below.

Liposomes

DNA incorporated into liposomes can be effectively introduced into living cells[12], and is the basis for at least one cystic fibrosis gene therapy trial, and at least one cancer gene therapy trial.

Jet injection

Mechanical devices which use air pressure to physically blast solutions into people have been used for vaccinations. This solution can actually penetrate several centimeters into tissue without significant damage. When DNA is introduced, low efficiency expression can be found along the path of injection.

Direct intramuscular or intravenous injection

Protective vaccination against lethal doses of influenza virus has been induced by direct intramuscular injection of recombinant plasmid DNA[13]. DNA can be expressed after intravenous administration as well[14].

Particle gun

DNA precipitated onto micron-sized gold particles can be propelled at high velocities into living cells and tissues and induce efficient expression[15]. These particles penetrate no more than 10 or so cell layers deep, but induce nearly 100% expression on the surface of the tumor or tissue.

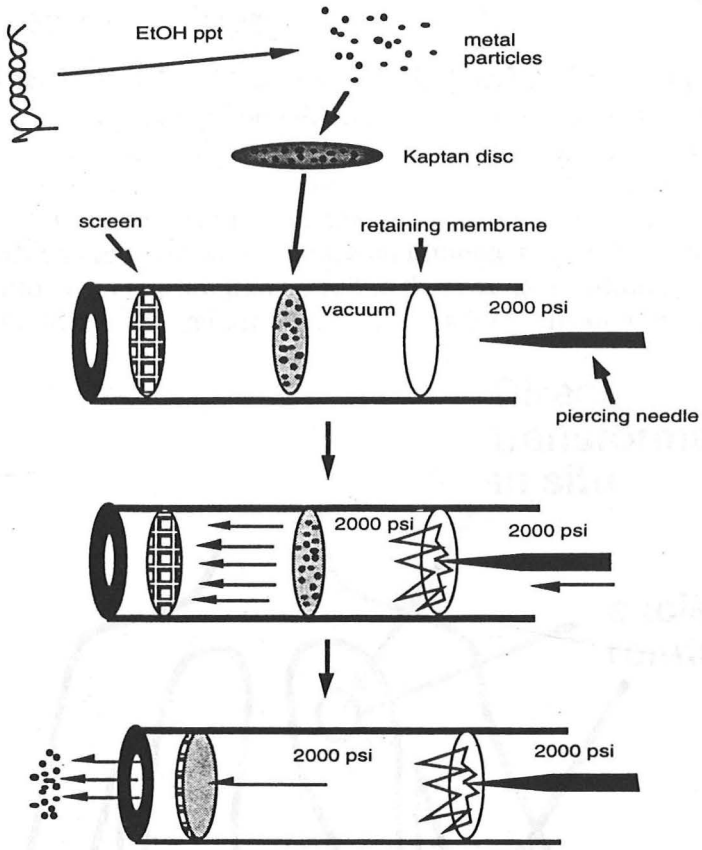


Figure 5. Schematic representation of the particle gun. Plasmid DNA containing the gene of interest driven by the desired promoter is precipitated onto micron -sized gold particles and these are dried onto a Kaptan disk. This disk is propelled by high pressure nitrogen and slapped against a wire mesh, which stops the disk, but allows the particles to pass through and into the tissue or cells in their path.

oligonucleotides

Short oligonucleotides can be directly taken up by cells and affect expression of endogenous genes. The half-life of these oligonucleotides can be prolonged by sulfur modification of the DNA to form phosphorothioates.

Vector delivery

General approaches to gene therapy

Depending upon the type of gene, the type of tumor, and the hoped for effect, tumor cells can be exposed to novel genes in any vector either *ex vivo* or *in vivo* (Figure 6). Genes which are directly tumor inhibitory are generally delivered to the tumor *in situ* in the organism, and those genes which are intended to induce an immune response or are being introduced into host cells are transferred to these cells in culture, and then the gene-modified cells reintroduced either as live cells or as a vaccine.

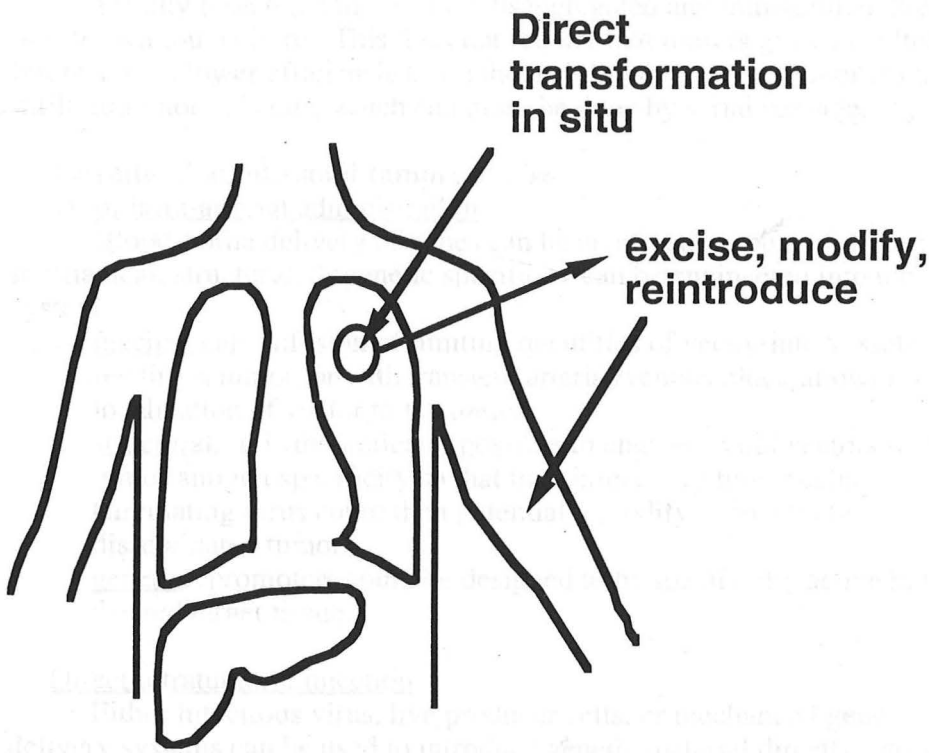


Figure 6. Therapeutic genes can be delivered *in vivo*, or into tumor or host cells *ex-vivo* and then re-introduced.

Modes of gene transfer

***In vitro* selection after stable transformation**

Coexpression of a selectable marker such as neo allows *in vitro* selection of clones of cells stably genetically modified. This allows the development of a clonal, genetically modified population of cells expressing the recombinant gene. This is good only for transduction of cells capable of

clonal growth, and most human tumors, for example, frequently cannot be cloned or selected in this manner. This is good for model systems.

Mass transformation/infection of short term cultures without selection

Most tumors can be coerced to grow for a few passages in culture, enough for retroviral vector integration and expression. In addition, many people believe that addition of a selectable marker to a recombinant retrovirus reduces the titers and expression levels of the other recombinant genes. This approach does not require such a selectable marker.

Introduction into solid tumor explants without culture

Freshly resected tumor can be disaggregated and transformed directly *in vitro* without culture. This does not require that tumors grow in culture, but results in lower efficiencies, and the inability to separate tumor from infiltrating normal cells, which can often be done by serial passage.

Introduction into solid tumors *in vivo*

IV or intra-arterial administration

Blood-borne delivery of genes can be an effective route of delivery IF mechanical, structural, or genetic specificity can be engineered into the system.

mechanical: infusion of limiting quantities of vector into vessels feeding a tumor, or with transient arterial/venous block allowing localization of vector to the tumor.

structural: it is theoretically possible to engineer viral vectors with tumor antigen specificity so that they infect only tumor cells. Circulating virus could then potentially modify even widely disseminated tumor.

genetic: promoters could be designed to be specifically active in the desired target tissue.

Direct intratumoral injection

Either infectious virus, live producer cells, or mechanical gene delivery systems can be used to introduce genetic material directly into solid tumors. This usually results in highly localized expression, but a relatively low fraction of the tumor cells expressing the desired gene. The injection of live producer cells is particularly effective for *in situ* retroviral transduction, as the particles are unstable and require target cell replication for stable integration. The producer cells bathe the targets in retrovirus continually for as long as they are functioning.

Which genes?

We have discussed vector and gene delivery systems, but the most difficult question remains, and that is what genes are the most effective and

intervention into which cellular pathways results in the best anti-tumor effect.

Genes with direct anti-tumor action

These are genes which either directly inhibit tumor growth, or make them sensitive to otherwise non-toxic agents. These genes have a readily explainable effect only on tumor cells which carry the novel gene.

A "bystander effect" is spoken of in the literature, which refers to killing of cells "near" the cells killed by receiving the gene. One study suggests that this is mediated by diffusable factors, but its reality and mechanism are unclear.

"Suicide" genes

Certain genes are non-toxic by themselves, but impart sensitivity to a drug upon cells expressing them. The best example is the Herpes Thymidine kinase gene. This gene from Herpes Simplex Virus is much more capable of phosphorylating drugs such as Gancyclovir to toxic compounds which kill cells able to perform such a conversion. This is the basis of its anti-viral action. If the HSV TK gene is introduced into tumor cells in an animal, and then the animal is treated with Gancyclovir, the cells expressing the TK gene will be selectively killed[16].

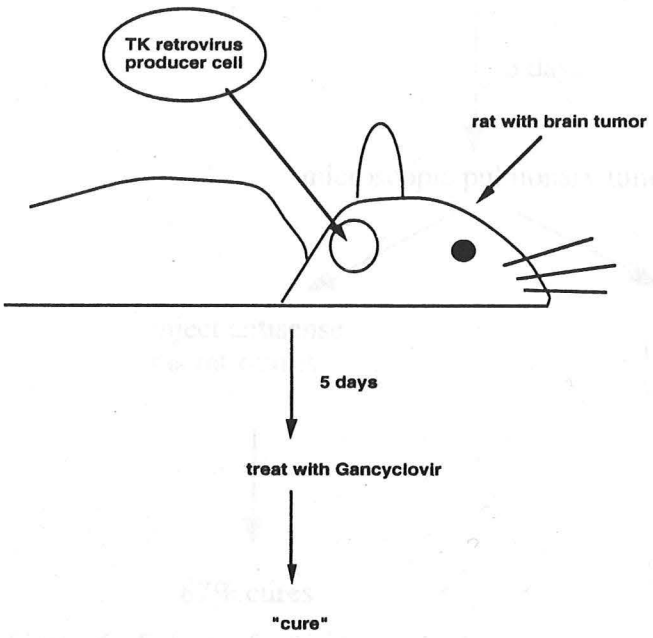


Figure 5. Direct *in vivo* instillation of retroviral producer cells making a recombinant retrovirus carrying the Herpes Simplex Virus Thymidine

Kinase gene confer gancyclovir sensitivity to tumor cells in a rat brain tumor model[16].

In one protocol[16], producer cells infected with a recombinant retrovirus containing the HSV TK gene are directly injected into brain tumors. This technique exploits the inability of retroviruses to infect non-dividing neural cells while efficiently infecting dividing brain tumor cells. There is a human clinical trial being initiated with this approach by Ken Culver and Mike Blaese, utilizing rodent producer cells stereotactically injected into human glioblastoma multiforme.

Antisense expression constructs

Mutant or overexpressed oncogenes are thought to be important in the maintenance of a tumor's aberrant growth. One gene-therapeutic approach is to introduce an antisense copy of the oncogene of interest and produce an antisense messenger RNA which anneals to the endogenous transcript forming untranslatable double stranded RNA. This blocks translation of the endogenous message[17, 18].

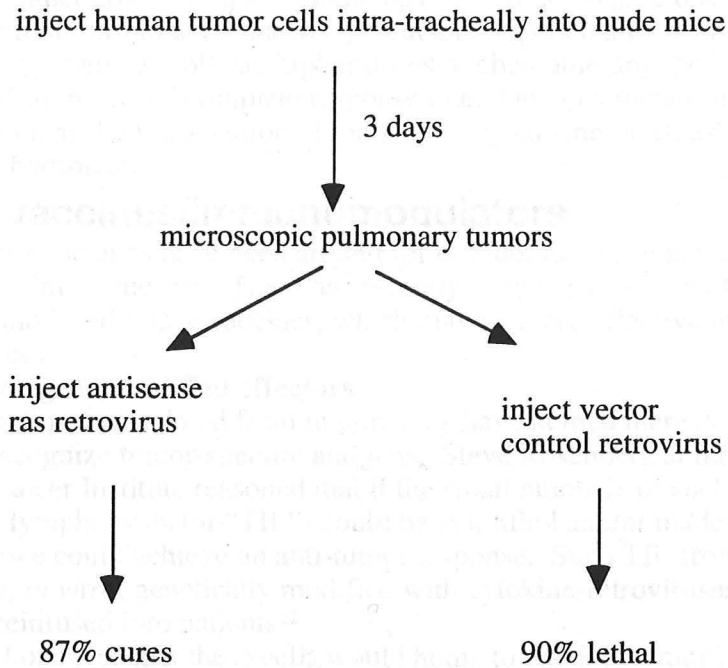


Figure 6. Schema for *in vivo* gene therapy using antisense *ras* retrovirus[17]. Human lung cancer cells carrying a mutant *ras* gene were instilled

intratracheally into nude mice. 3 days later, antisense *ras* retrovirus was introduced by the same route.

The fact that a single instillation of the retrovirus, which infects only a fraction of the tumor cells, results in a significant number of "cures" is evidence for a "bystander effect" *in vivo,* or killing of non-gene-modified tumor cells which are "near" gene-modified ones. Host immune responses (in this case NK cells) may also play a role. This experiment is the basis of a clinical trial approved by the RAC in human lung cancer to be conducted at the MD Anderson hospital[19]. In this study, 14 patients who are ineligible for surgery, radiotherapy, or chemotherapy and have non-small cell cancer obstructing a bronchus, but reachable with a bronchoscope, will be treated with direct intra-tumoral injection of a retrovirus bearing an antisense *ras* gene.

Genes with host-protective function

Overexpression of the membrane pump protein "MDR" can induce resistance to certain classes of chemotherapeutic agents. When this gene is introduced into transgenic mice, the hematologic precursors from these mice are found to be resistant to the effects of these drugs, which allows the mice to survive higher doses of the chemotherapy[20, 21]. It is possible that genetic modification of normal bone marrow precursors in patients with cancer would allow patients to tolerate higher doses of chemotherapy, potentially resulting in an increased complete response rate. Other examples are possible, such as the introduction of metabolizing enzymes such as bleomycin hydrolase.

Tumor vaccines/immunomodulators

Tumor vaccines have been around for decades and have not acheived significant clinical success. There has recently been a resurgence of interest with gene-modified tumor vaccines, which may be more effective in some circumstances.

Cytokine gene-modified effectors

Lymphocytes isolated from tumors may have homed there due to their ability to recognize tumor-specific antigens. Steve Rosenberg at the National Cancer Institute reasoned that if the small numbers of such tumor infiltrating lymphocytes (or "TIL") could be amplified and/or made more cytotoxic, one could achieve an anti-tumor response. Such TIL from tumors were grown *in vitro*, genetically modified with cytokine-retroviruses such as TNF, and reinfused into patients[22].

The hope was that these cells would home to residual tumor in the patient and eradicate it. Unfortunately, only a tiny fraction of the re-infused cells returned to tumor deposits, less than 1%, and this was insufficient for clinical efficacy.

Cytokine gene-modified tumor

Much recent interest has been generated from the finding that certain tumors became non-tumorigenic when transduced with a cytokine gene, and importantly, became immunogenic. After exposure to such gene-modified cells animals become immune to non-gene modified tumor cells by an unclear mechanism. Thus a vaccine can be prepared by genetically modifying autologous tumor cells with cytokine genes, radiating them to prevent them from growing, and re-injecting them as a vaccine. A large variety of cytokines have been introduced into a variety of animal model tumors with variable success. These include IL-2[23-26], IL-4[26-28], IL-6[29, 30], IL-7[31, 32], TNF[33, 34], IFN-γ[35, 36], and GM-CSF[37].

One study introduced an expression vector for murine IL4 into the murine renal tumor RENCA, and found not only that they had lost tumorigenicity, but were able to induce a systemic CD8+ cytolytic immunity to non-gene-modified parental tumor cells[27]. The authors of this study did not use radiated parental cells as negative controls, and it has subsequently been stated that the same result can be achieved by vaccinating with such cells.

More extensive studies were undertaken and in the B16 model, GM-CSF was found to be the most potent gene to induce systemic immunity, even though it did not cause loss of tumorigenicity[37]. If GM-CSF-modified tumor cells were injected into syngeneic mice the mice died of their tumors, but if irradiated GM-CSF-producing cells were injected, a systemic immunity was produced, which in this case was not elicited by irradiated tumor cells alone. This immunity reacted with non-gene-modified cells. These experiments are the basis for an approved human gene therapy clinical trial in renal cell cancer using GM-CSF retrovirus.

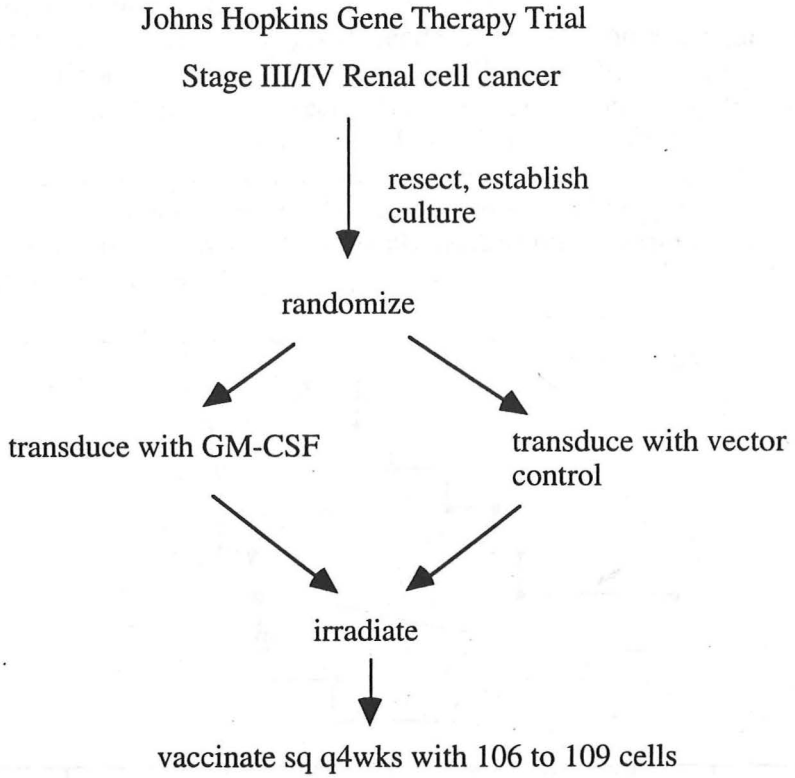


Figure 7. Schema for the Johns Hopkins cytokine gene therapy clinical protocol (Jonathan Simons, personal communication).

The new cytokine IL12 has recently been found by Lotze et al to be a very potent inducer of this effect (personal communication).

If one looks at gene-modified tumors histologically, different cytokines cause infiltration of different cell types: IL-2 producing tumors infiltratedby lymphocytes and neutrophils, IL-4 producing tumors infiltrated by eosinophils and plasma cells, and gamma interferon producing cells infiltrated by macrophages.

Fundamentally, it is not known how these cytokines are causing this increased immunogenicity, and or fact whether it is a phenomenon which is seen only in highly selected circumstances and of little real utility.

Class I MHC molecules

Transfection of allogeneic class I MHC molecules can cause an increased immunogenicity and rejection of modified tumors[12]. This is also being tested in human clinical trials using the human HLA B7 gene.

Co-stimulatory molecules

Target recognition is not solely dependent on MHC molecules and T cell receptors, but other molecules, such as B7/CD28 are required for efficient recognition. An exciting recent finding is that tumors may escape immune recognition by failing to express B7 on their cell surface. Transfection of the B7 molecule can both abrogate tumorigenicity and induce protective immunity, since MHC class I restricted killing is independent of the presence of B7 - it is only needed for the priming, not action of cytotoxic T cells[38, 39].

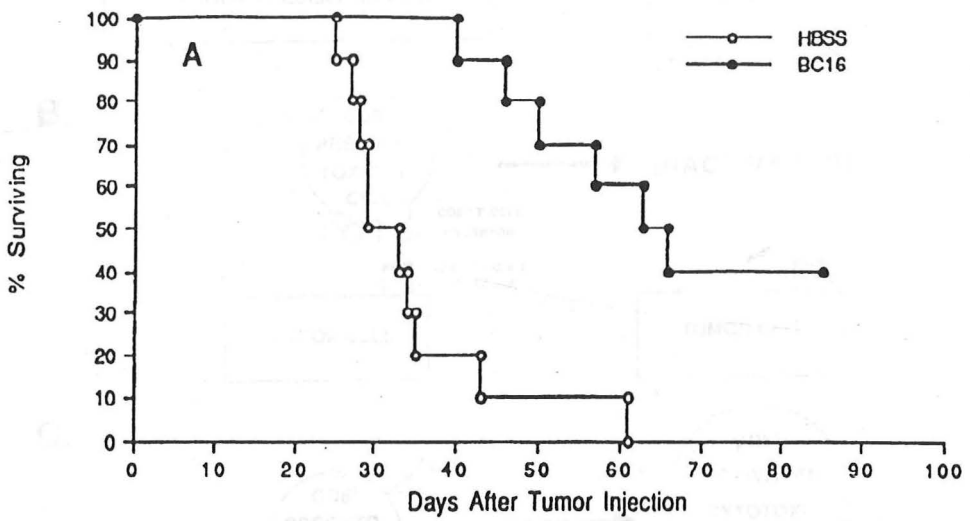
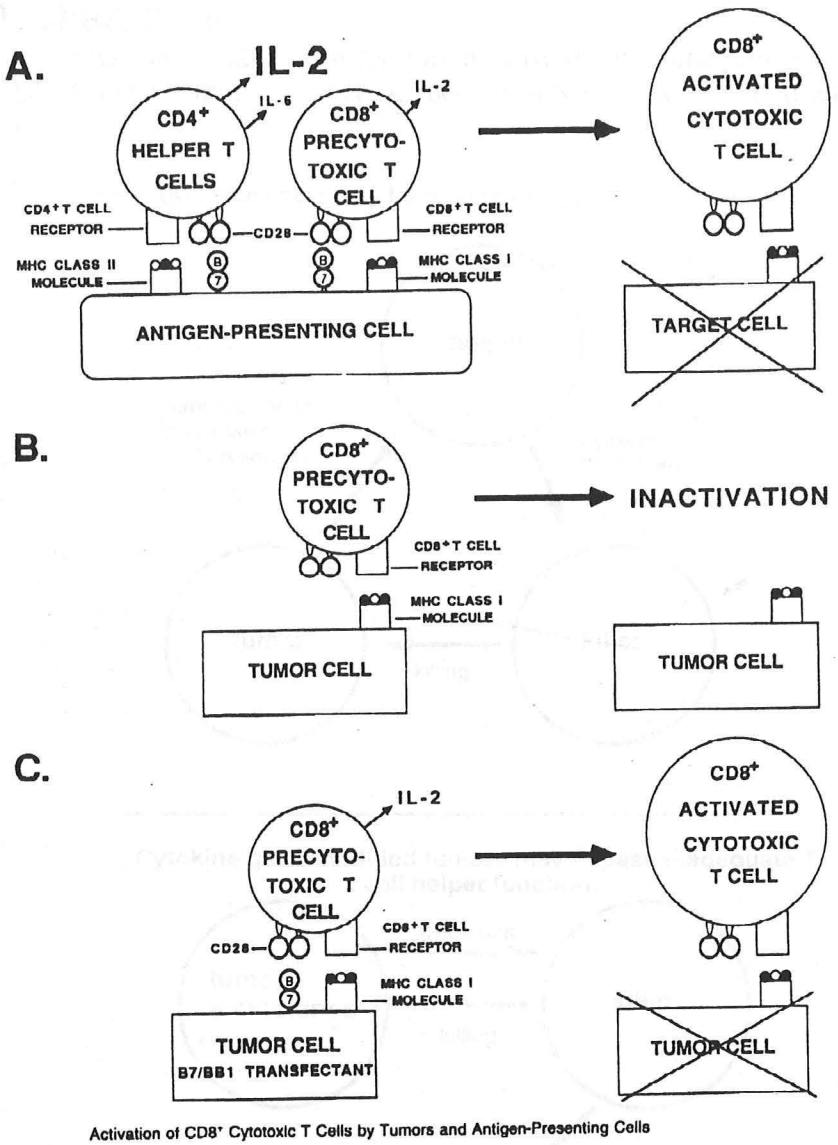


Figure 8. Elimination of pre-existing metastatic parental (B7-) cells by subsequent intravenous injection of B7 modified tumor. Approximately 40% of the animals are cured. Figure adapted from [39].



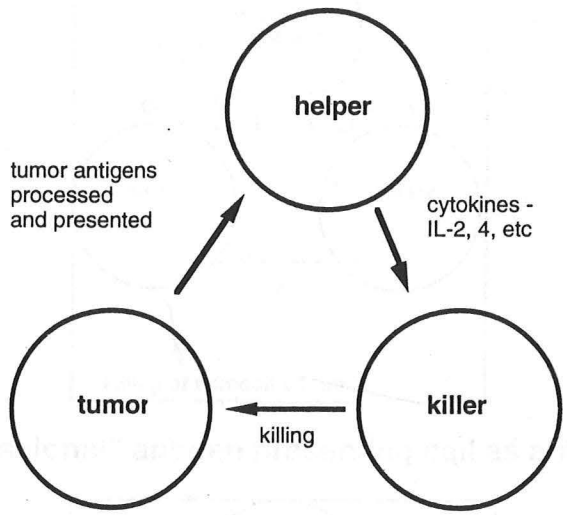
Activation of CD8+ Cytotoxic T Cells by Tumors and Antigen-Presenting Cells

Figure 9. Model for tumor escape from immune recognition by lack of B7 expression, and the return of a response by the artificial introduction of the gene. T cells primed by the gene-modified tumor recognize non-gene modified tumor that is not expressing B7.

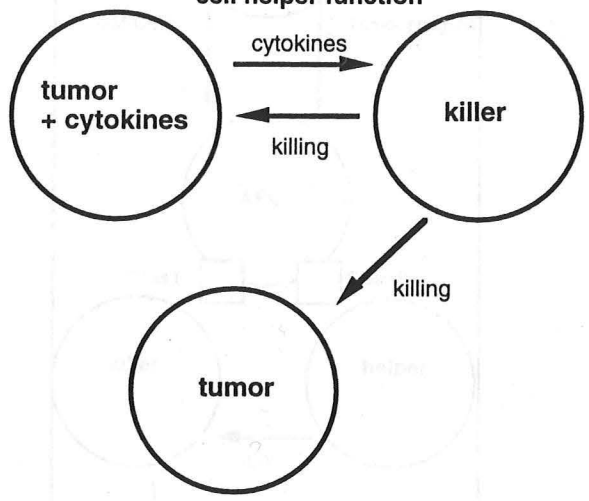
Mechanisms

How can cytokine transfection increase the immunogenicity of tumor cells? There are multiple possible mechanisms, outlined in the figures below.

Effective T-cell help may be required for tumor cell killing

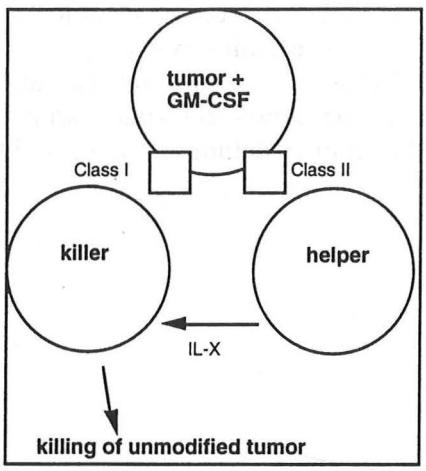


Cytokine gene-modified tumors may bypass inadequate T cell helper function

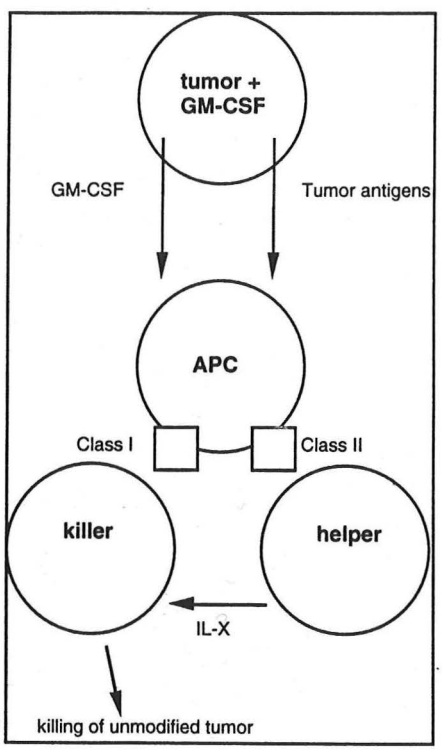


What is the antigen-presenting cell in the cases where immunity is elicited? There are several models: the tumor cell itself, or antigen presenting cells.

1) The tumor cell as antigen presenter



2) A "professional" antigen presenting cell as antigen presenter.



Future Issues and problems

- new targeted vectors: engineered viral receptors for tumor-specific delivery of genes
- new genetic targets, "combination" gene therapy
- inconsistent results with different cytokines. Some authors report that gene-modified tumor cells are no better than tumor cells in adjuvant[40].
- cytokine production by tumors sometimes increases metastatic capacity and lethality - is *in vivo* cytokine therapy safe?
- is there a common mechanism of increased immunogenicity that can be exploited (cytokines plus costimulatory molecules?)

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