Transcriptional Regulation in Cancer Gene Therapy

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Gene therapy for cancer aims at cell killing. Delivery of a functional tumor suppressor gene may repress tumor growth [1], but genetic alterations that result in a malignant phenotype are so complex that elimination of these clones is probably required for cure. Currently, there is already a battery of genes demonstrated to have an efficient killing effect on various tumor cells. These genes should be expressed at the right site, at the right time and at the appropriate level. Therefore, control over the delivery and expression of powerful lytic genes requires adequate regulatory mechanisms. Therapeutic targeting to malignant tissues may be extrinsic or intrinsic [2]. Extrinsic targeting implies the local effect of surgery, irradiation, or drug delivery to tumors. Intrinsic targeting involves transductional targeting (selective uptake of vector) or transcriptional targeting (selective expression of transgenes).

In transductional targeting the interaction between the vector and the cell surface determines the efficiency of targeting. As a proof of principle, adenoviral surface targeting of the pancarcinomal membrane antigen results in efficient cancer gene therapy [3], rather than non-productive binding. Tumor-selective transduction may also use protease-rich tumor cell surfaces [4], modification of viral envelope protein sequences, pseudotyped viruses, and antibodies as mediators for viral infection to restrict viral infection to specific cell types [5]. Transductional targeting for cancer may also successfully aim at the tumor vascular supply rather than at the tumor itself [6].

Following successful transductional targeting, expression of the foreign gene must be regulated (also referred to as transcriptional targeting or targeted gene expression). Transcription of the gene of interest will only occur under intracellular conditions in which the tissue-specific promoter is activated. Hence, regulatory signals are mandatory for controlled expression of therapeutic genes. These signals aim both at constitutive and inducible elements in the host genome, and may involve both the target tissue and adjacent tissues. The present discussion will focus on regulating promoter activity since other techniques of blocking gene transcription, such as gene disruption, do not usually affect more than 1 in 1,000 cells [7]. Gene disruption for cancer therapy is performed with targeted in-frame insertions within exons by way of homologous recombination. It aims at deleting dominant oncogenes, disrupting genes encoding for carcinogen-metabolizing enzymes [8], or at genes encoding for peptides responsible for tumor propagation [9].

Construction of viral vectors harboring cell-specific promoters has been applied for controlling gene regulation. While transcriptional regulation can generally restrict the expression of the therapeutic sequence to appropriate cells [10], promoters for genes typically expressed in a subset of malignant cells have been shown to direct therapeutic genes into the corresponding classes of tumor cells [11]. These promoters may be classified as nucleotide sequences that are already expressed by the cell, or as sequences that are not regularly expressed and may be subject to external stimuli. Regulation of therapeutic gene expression within tumor tissue may be classified into these groups: a) recognition of tissue-specific promoter sequences by distinct cellular transcription factors (trans-activators), b) exploiting oncogenes or tumor-associated peptides to construct their promoters with therapeutic genes, and c) up-regulation of protective genes in response to stress.

Tissue-specific promoters

Liver tissue

Hepatocytes may selectively express transgenes linked to the promoters of the gluconeogenesis enzyme PEPCK and the α1-antitrypsin protease. A segment of the PEPCK promoter is active in hepatocellular carcinoma and is regulated by insulin, glucocorticoids and cAMP. *In vitro* targeting of hepatocellular carcinoma was accomplished with α-fetoprotein and carcinoembryonic antigen promoters [12]. CEA is a common tumor marker expressed in colon, hepatic and lung carcinomas. However, it may also be expressed in benign inflammatory conditions, albeit less abundantly. The CEA promoter was attached to suicide genes, such as thymidine-kinase (*tk*) or cytosine deaminase (*cd*). The introduction of CEA sequence upstream of the *cd* gene has been shown to selectively sensitize malignant cells to 5-fluorocytosine cytotoxicity. CEA promoter was also used *in vivo* to drive *cd* expression in tumor xenografts and convert 5-FC to 5-fluorouracil. α-fetoprotein is another relatively specific gene expressed by hepatocellular carcinoma. Its promoter has also been successfully linked to *tk* and cloned

CEA = carcinoembryonic antigen
5-FC = 5-fluorocytosine

into a retroviral vector to infect and selectively kill hepatoma cells.

Hematopoeitic system

B lymphocytes, transfected with constructs of promoter and enhancer of immunoglobulin heavy chain and the diphteria toxin gene, show selective toxic expression [13]. A cytotoxic effect on T lymphoma cells and human cervical carcinoma cell line may be achieved by engineering an adenovirus vector harboring the herpes simplex virus *tk* gene regulated by human immunodeficiency virus long terminal repeats, used as a promoter. The gene was expressed and caused efficient cell killing after exposure to gancyclovir [14]. Interestingly, this strategy was also used to target suicide gene expression to HIV-infected lymphocytes [15].

Inverse targeting may prove ideally suited to address bone marrow suppression by chemotherapy. Retroviral vectors may allow selective transduction of receptor-negative cells (or non-transduction of receptor-positive cells) in a mixed cell population. Inverse targeting strategy is useful for carcinoma and hematopoietic cell mixtures. It may serve two goals: a) transduction of carcinoma cells (often displaying high density of epidermal growth factor receptors) with oncolytic gene while sparing the hematopoietic cells (lacking EGF receptors), and b) selective transduction of hematopoietic cells with *MDR-1* gene (encoding the efflux protein p170), while exposing carcinoma cells to chemotherapy [16].

Breast carcinoma

The mammary tissue is unique in expressing β-casein and acidic whey protein genes, harboring repressor elements within their promoters, and negatively regulating non-mammary tissues. The expression of milk proteins is regulated by complex promoters, having both positive and negative effects on gene expression, under hormonal regulation [17]. Since breast carcinoma is often a long-lasting disease with metastatic spread, identification of tissue-specific promoters is of paramount importance in order to deliver oncolytic genes systemically. Gene therapy for breast carcinoma may be approached by tailoring a virus with affinity to this tissue, such as the mouse mammary tumor virus. The glucocorticoid-responsive long-term repeats of this retrovirus were used as promoters for dexamethasone-inducible oncolytic cytokine expression. In clinical trials of recurrent breast carcinoma expressing *HER2* gene [18], patients were transfected with a plasmid containing *cd* gene driven by the AP-2 promoter. The efficiency of the cell killing following pro-drug activation was proportional to cellular *HER2* expression.

Prostate carcinoma

Prostate carcinoma cells may selectively express the toxic gene by activating a gene located downstream of the prostate-specific antigen. This promoter positively responds to insulin-like growth factor-1 and EGF. Prostate tissue specificity was also induced by the use of androgen-dependent PSA promoter [19], and increased efficiency was achieved by coupling the PSA promoter to a yeast promoter [20].

Malignant melanoma

Malignant melanoma specificity may be conferred by the human tyrosinase promoter, specific to melanoma cells. *In vivo* TYR-GALV plasmid transduction of a melanoma tumor resulted in expression of fusion proteins and complete tumor regression, while this transcriptionally regulated plasmid did not affect other tumors. Melanoma-specific targeting with tyrosinase promoter was demonstrated earlier *in vitro* [21]. Melanoma often expresses metalloproteinases abundantly. Adenoviral-mediated tissue-specific gene delivery of tissue inhibitors of metalloproteinase significantly reduced local invasion *in vitro* [22], possibly by the induction of apoptosis.

Central nervous system

Gene therapy of the brain is hindered by the presence of the blood-brain barrier, mandating invasive routes of administration. Immunoliposomes have been shown to deliver reporter genes in rats by targeting the plasmid DNA conjugated to BBB transferin receptor monoclonal antibodies [23]. While gene expression was shown throughout the central nervous system, proving the feasibility of brain transductional targeting, transcriptional regulation has yet to be shown. Neuroectodermal specific promoters include calcineurin A-α and synapsin-I. Use of these promoters may limit oncolytic gene expression to brain tumors often addressed by immunotherapy-based gene therapy. Transgenes potentially regulatable that have already been transduced to brain tissue include β-interferon [24] and macrophage colony-stimulating factor [25].

Tumor-specific promoters

Other than tissue-specific promoters, transcriptional regulation of therapeutic genes for cancer may aim at tumor-associated genes. Viruses may use the intrinsic properties of the transformed cells as the basis for tumor specificity. Deletion of the early replication E1B gene selects this adenovirus mutant to replicate preferentially in non-functional p53 malignant cells. The E1B-deleted ONYX-015 virus showed a beneficial clinical effect in patients with recurrent head and neck carcinomas, by selectively infecting the tumor cells [26]. This virus does not have a therapeutic transgene but relies on the lytic effect of the adenovirus [Figure 1]. Preferential replication for specific tumor cells was also reported for reovirus, infecting tumor cells with

5-FU = 5-fluorouracil
HIV = human immunodeficiency virus
EGF = epidermal growth factor
PSA = prostate-specific antigen
BBB = blood-brain barrier

activated Ras pathway [27] and the prostate-attenuated replication-competent adenovirus.

Oncogenes are genes involved in the malignant transformation. These genes include *c-erb*B2*, and c-myc*. The *c-erb*B2 oncogene is involved in some types of breast, gastric and pancreatic carcinomas. Its promoter is activated in these tumors by the trans-activator OB2-1. This feature was exploited for constructing this promoter with a *cd* gene to selectively kill breast and pancreatic cell lines overexpressing *c-erb*B2. A similar approach using *c-myc* was applied for small cell lung carcinoma *in vitro* [28].

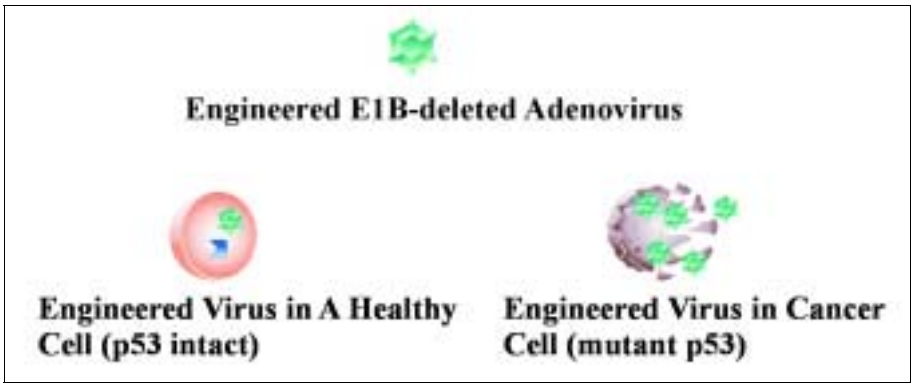

Figure 1. Replication of an engineered adenovirus within a p53 mutant tumor cell. The intact p53 tumor suppressor product inhibits the replication of the mutant virus within a normal cell, whereas deletion of the E1b adenoviral gene may selectively direct its replication to the p53 deficient tumor cell.

A common feature of head and neck, lung, breast, colon, ovarian, endometrial and bladder carcinomas is the expression of the secretory leukoproteinase inhibitor. Its promoter was constructed with the *tk* gene to selectively kill lung and ovarian carcinomas.

Antisense oligodeoxynucleotides harbor a potential for highly specific gene targeting. ODN are short sequences of complementary DNA synthesized exactly to complement specific mRNA. As RNA-DNA hybridize, message translation is interrupted. The potential advantage of anti-oncogene therapy rests in selectivity for mutant cells while sparing normal cells. Targeting *bcr/abl* transcripts after the juxtaposition by translocation has occurred suppresses Philadelphia leukemic cell proliferation in chronic myelogenous leukemia cell lines [29] and SCID mice survive longer [30]. Two distinct ODN, targeting both *bcr/abl* and *c-Myc,* a second oncogene involved in signal transmission in CML, were more potent in reducing leukemic cell load. *c-Myb* antisense ODN targeting has also been shown to inhibit growth of various leukemic cell lines, depending on this gene for proliferation [31]. Clinical experience with *c-Myb* and *bcr/abl* antisense ODN for CML phase I trials showed that this therapeutic modality is safe, while *bcl-2*-targeted ODN led to a slight improvement in a limited number of patients with non-Hodgkin's lymphoma. Recently, human non-Hodgkin's lymphoma was eradicated in SCID mice by *bcl*-2 antisense oligonucleotides combined with low dose cyclophosphamide [32]. Antisense ODN have also been produced to target synthesis of tumor-endogenous immunogenic-masking proteins, such as IGF-1 and transforming growth factor-β [33].

ras proto-oncogenes are found in various human cancers and result in the p21 protein, possibly a tumor-specific antigen, thereby potentially presenting a tumor-specific antigen for the immune system [34]. As malignant tissues are often non-immunogenic as a result of down-regulation of surface major histocompatibility expression and antigen display, oncogene-specific vaccines may be developed only if tumor-specific T cell clones can be generated with high selectivity for neoantigen recognition.

Stimulation of tumor immunity may be aroused by killing tumor cells in a way that will disperse its specific intracellular antigens and present it to antigen-presenting cells. Cell death induced by necrosis rather than apoptosis may be associated with stress that sensitizes the immune system for a danger signal.

Inducible promoters

The third group of promoters suitable for gene expression regulation in tumors are stress-gene promoters. Heat, hypoxia, glucose deprivation, irradiation, antibiotics and chemotherapeutic agents, all up-regulate genes involved in these stress-response conditions. In this respect, because cancer treatment often induces stress, gene regulation may be driven by coupling the therapeutic gene to a stress-gene promoter. The stress genes up-regulated in these conditions include *MDR-1* (multi-drug resistance gene), *HSP* family (human heat shock [stress] protein gene), and the irradiation-inducible *Egr-1* (early growth response gene), *WAF1* and tissue plasminogen activator (*tpa*) promoters.

Irradiation-responsive promoter sequences were identified for tissue plasminogen activator, *Egr-1*, and *WAF1* genes. The first radiation-inducible promoter system used in combination with gene therapy involved the *Egr-1* promoter with the gene for the radiosensitizing cytokine tumor necrosis factor, which resulted in increased tumor growth inhibition compared with tumors treated with radiation alone. *Egr-1* promoter coupled to the gene for HSV*tk* produced enhanced tumor cell killing in the presence of the pro-drug ganciclovir following radiation treatment [35].

The heat shock (stress) protein family is induced by a variety of environmental stresses, namely heat, irradiation, photobeam

ODN = oligodeoxynucleotides
CML = chronic myelogenous leukemia
IGF-1 = insulin-like growth factor

irradiation, hypoxia, acidosis, hypoglycemia, and osmotic changes. These conditions may exist in poorly vascularized tumors and may trigger expression of anti-cancer genes linked to the HSP70 promoter. Importantly, in p53-deficient tumor cells, HSP70 is up-regulated, resulting in enhanced selection of gene expression [36]. The HSP70 promoter served *in vivo* to selectively kill tumor cells and spare normal cells where p53 protein represses HSP70 promoter. The *GRP78* gene is a member of the heat shock (stress) protein family and its promoter has been used to efficiently activate reporter genes [37].

MDR-1 encodes a membrane-effluxing glycoprotein whose expression is induced by vincristine, actinomycin D and doxorubicin. Its promoter is indirectly transactivated by these compounds and induces transcription and expression of therapeutic genes, such as TNF-α in tumors exposed to chemotherapy [38]. Chemotherapy also induces other mechanisms for drug resistance, namely activation of the glutathione detoxification system and apoptosis-controlling gene alterations (especially p53 and bcl-2).

Switch-off mechanisms

The above conditional expression constructs are attractive because they depend to a large extent on the biology of the tumor, or are already induced by various therapeutic modalities. Interestingly, transfection itself may induce wild-type p53 activity and limit the spread of genes that are expressed in mutant p53-tumor cells. However, a switch-off mechanism for these systems is necessary to decrease the risk of damage to normal tissues. This may be achieved by various means.

The tetracyclin repressor

The TetR is a tetracycline-inducible repressor that can be used to efficiently repress gene expression in mammalian cells. TetR binds to the two tet operators, resulting in repression of both genes – the tetracycline/metal-proton antiporter located in the cytoplasmic membrane, and the regulatory protein TetR itself. The Tet-controlled transcription system is comprised of Tet-Off and Tet-On transcriptional regulation, derived from the *Escherichia coli* Tet-resistance operon. The trans-activator (tTA) consists of a fusion of wild-type Tet repressor and the herpes simplex VP16 activator domain. In the Tet-Off state, tTA binds the Tet repressor element and activates transcription in the absence of tetracyclin or doxycyclin. The Tet-On option was available following substitution of four amino acids at the Tet repressor, thereby altering its binding characteristics and creating reverse repressor (rTetR). This protein binds the tetracycline-responsive element in the presence of doxycyclin and activates transcription. Simultaneous expression of two distinct genes is possible under the control of a single tetracycline-responsive element.

The Tet-R system can be used to suppress and induce cytotoxic gene and reporter gene expression. The latter selected gene expression to p53-deficient tumor cells and documented the feasibility of a second-line control, i.e., induction of mRNA antisense by p53-dependent trans-activation in normal cells. However, the specific nature of any antisense RNA may limit the spectrum of gene insertion into the vector. On the other hand, the transcription repressor system may enable substitution of therapeutic genes.

Antisense insertion

Intracellular molecular control over a wide array of genes may be achieved with antisense insertion. Unlike primary oncogene targeting with an antisense ODN, this system involved co-transfection of a second construct. A repressor of the therapeutic gene can be put under the control of a promoter, activated by wild-type p53 expression in normal cells and expressing a transcriptional repressor or an antisense for the therapeutic gene.

Table 1. Transcriptional regulation of cancer gene therapy

Promoter	Tumor	*In vivo*	Therapeutic gene	Ref.	Inducer
Tissue-specific					
Thyrosinase	Melanoma	Yes	FMG, HSV-tk, IL-2	11	
CEA	Colon, liver, lung	Yes	HSV-tk, HSV-cd	13	
Ig heavy-chain	B lymphoma	No	Diphteria toxin	13	
AP-2	Mammary	Yes	HSV-cd	18	
PSA	Prostate	No	Polyglutamine	20	
Tumor-specific					
E1B-deleted adenovirus	p-53 mutant	Yes	None	26	
Erb	Mammary, pancreas	No	HSV-cd	18	
Myc	Lung	No	HSV-tk	28	
Inducible					
Egr-1	Brain	Yes	TNF-α	35	Irradiation
GRp78 (Hsp family)	Fibrosarcoma	Yes	Reporter	37	Hypoxia, acidosis
MDR1	Mammary	Yes	TNF-α	38	Chemotherapy
Hsp70	Prostate, skin	No Yes	HSV-cd and tk	36	Hyperthermia, hypoxia, photobeam, hyperosmolarity

FMG = fusion membrane glycoproteins, MDR = multiple drug resistance, Hsp = heat shock protein, Ig = immumnglobulin, tk = thymidine kinase, cd = cytosine deaminase, HSV = herpes simplex virus.

TNF = tumor necrosis factor
TetR = tetracycline repressor

Conclusions and prospects of conditional gene expression

Gene therapy confronts not only efficacy of gene delivery but also control of its expression [Table 1]. Regulation is of major priority, especially in cancer gene therapy where expression of powerful lytic genes may cause diffuse normal tissue damage. The three levels of gene regulation discussed above include tissue specificity, tumor selectivity, and activation by a stress signal induced by cancer therapy. These formulas for control over genes exploit either the unique tissue or the aberrant tumor phenotype, or construct particular promoters that regulate gene expression under specific conditions. In addition to control over transgenes, transcriptional targeting may also enhance efficiency of gene expression by avoiding the universal host immunogenic response to the viral vector, as promoter specificity and tissue specificity may determine host immune response to the transgene.

Among the other recently described novel cancer gene therapy modalities are the preferential growth of bacteria in tumors [39] and the homing of stem cells to brain tissue [40]. The latter may direct the expression of transgenes to gliomas, obviating some of the obstacles of transductional targeting.

Since the theme of gene therapy for cancer is increased efficiency with decreased toxicity, combining both improved promoter design and vector specificity should allow fine-tuning expression of therapeutic genes for cancer therapy.

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