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Tissue specific cancer gene therapy

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ABSTRACT

Ultimate goal to cancer therapy is to specifically eradicate tumor cells while normal tissue remains intact. In this regard, gene therapy protocol has large potential for the effective and specific cancer treatment. One advantage of cancer gene therapy over conventional anticancer drugs is to control cancer specificity of anti-cancer transgene activities with tissue and/or cancer-specific promoters. In this review, we mainly focus on recent endeavors to develop transcriptional targeting for cancer to increase specificity and effectiveness of cancer gene therapy. Although problem of promoter leakiness has not been totally excluded, recent technology advances to identify new cancer-specific genes and components and combination of various enhancer/promoter systems coupled with positive feedback loops give more opportunities of stronger and more specific transgene expression. With targeted gene delivery, transcriptional targeting of gene expression is one of important weapons to be equipped for successful genetic approach to tumor modulation.

Key words : cancer gene therapy, tissue-specific expression, tissue-specific promoter

Introduction

Gene therapy may some day prove to be a powerful modality in treating a wide range of nongenetic disorders. Incipiently, gene therapy is viewed as an approach for treating hereditary diseases, such as severe combined immune deficiency, familial hypercholesterolemia, cystic fibrosis, Gaucher's disease, and etc. However, its potential role in the treatment of acquired diseases such as cancer is now widely recognised. Cancer gene therapy requires to overcome a complex biological issue that generates specific risks as recently evidenced during the treatment of genetic diseases with adenoviral and retroviral vectors(1). It can be suggested that gene therapy is not toxic because gene therapy is not efficient. This opinion may hold some truth, but the use of increasing doses, the use of more complex strategies, and the one of more potent genetic activities are now leading us to reach the borderline between safety and toxicity, which make the biosafety a major determinant in the future of this therapeutical approach. We have to consider both the design of a gene transfer product and the possibility for this product to bypass the safety keys set up by the investigator to avoid the induction of unexpected deleterious effects. As patients' safety requires tumor selectivity, tight control over these agents is of paramount importance. In this regard, targeting transgene expression to cancer gene therapy is an area of intense research. Consequently, gene therapy offers the potential for enormous improvements in the targeting of cancer therapy, but this has not yet been achieved, although early trials are promising.

As cancer therapies set on accomplishing the safety, one of the main trials for the cancer gene therapy is the selective targeting and killing of tumor cells, thereby increasing the therapeutic ratio. The therapeutic index of currently available modalities for most cancer is low. Consequently, cancer pa-

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tients often witness severe adverse effects, most notably myeloid stem cell suppression. Available cytotoxic agents have a cancer cell-to-normal cell therapeutic ratio as low as 2:1 to 6:1. Therefore, innovative strategies for the treatment of cancer are highly desired. Recently, novel strategies using gene therapy strategies have been reported to achieve tumor-selectivity from 100:1(2) to 10,000:1(3). Both chemotherapy and radiotherapy induce dose limiting normal tissue toxicities, which reduce their clinical effectiveness. Cancer gene therapy has the advantage that normal tissue toxicity might be avoided if suitable strategies can be employed to target the therapeutic transgene directly to tumor cells. Therefore, trascriptional targeting with tumor- and/or tissue-specific promotor/enhancers for the cancer cells is useful to achieve safety. Especially, transciptional targeting takes advatage of the fact that some cancer cells express a subset of exclusive genes, and uses these cancer-specific promoters to express the desired transgenes(4).

To date, as anther approach, clinical trials have focused on the delivery of genes directly to the tumor site by intratumoural injection using both viral and nonviral delivery agents, thereby largely avoiding normal tissues. However, the goal of most cancer gene therapy is able to administer a suitably packaged transgene systemically and achieve a high level of tumor targeting. This will be important for targeting the majority of tumors, which are not accessible to direct injection, and to ensure adequate distribution of the transgene throughout the tumor mass. The blood supply still offers the best opportunity to do this. Systemic delivery of transgenes would also allow targeting of both the primary tumor and metastatic deposits, which must be controlled if therapy is to be successful. A number of strategies are now being developed to target both viral and nonviral delivery agents to tumor cells. These include exploitation of natural viral tropisms, such as those exhibited by adenoviruses to target lung epithelium; retargeting viruses using a bispecific molecule to simultaneously block native receptor binding, redirecting virus to a tissue-specific receptor; genetically modifying the virus to ablate native receptor interactions and incorporating a novel ligand into one of the virus' coat proteins; using tissue-specific ligands or monoclonal antibodies incorporated onto the surface of liposomes to direct them to target cells(5).

As well as controlling the delivery of the therapeutic gene to the tumor tissue, controlled regulation of transgene expression is now playing a major role in targeted cancer gene therapy strategies. Indeed, by combining targeted delivery with tumor-specific expression, the level of transgene product in nontarget normal tissues, compared with that in tumors, can be greatly reduced. Therefore, current cancer gene therpay protocols mainly focus on transciptional targeting and the targeting of synthetic gene delivery. Therapeutic targeting to malignant tissues may be extrinsic or intrinsic(6). The purpose of this review is to focus on transcriptional targeting for cancer, and discuss strategies to amplify the magnitude of specific expression.

Transcriptional control

The production of protein within a cell requires that the appropriate gene be transcribed into mRNA and then translated into protein. This process is complex and subject to multiple levels of control. The regulation of transcription is the key initiating event in this process and is mediated by the interaction between the enhancer/promoter region of the appropriate piece of DNA and the specific proteins or transcription factors that bind to this region. For transcriptional targeting in cancer gene therapy, a promoter that is either tissue specific or tumor specific is used to drive expression of a novel transgene. Recently, advancements have been made in identifying certain tissue-specific promoters of utility for the creation of transgenic models of some solid tumor types and to yield tumor-specific transgene expression for the purpose of gene therapy.

Tissue specific targeting

Despite the fact that almost every cell in the body contains a complete copy of the genome, phenotypic heterogeneity is largely achieved through differences in the patterns of gene expression that are, in the main, controlled at the level of transcription. Activation or repression of promoters is achieved through interactions with specific transcription factors.

Consequently, some tissues express proteins that are specific to that tissue because the promoter for the appropriate gene is only activated in that tissue. Thus, the success of transcriptional targeting is dependent on differential expression of genes in cancer cells compared to normal cells. In other words, cell-specific expression can be thought of as being mediated by a unique subset of ubiquitous and specific activators present in the cell. Transcription targeting is feasible because the tissue- or cancer-specific promoter can be activated in the targeted cancer cell in the presence of the proper subset of activators but would remain silent in the non-targeted cell.

Liver specific promoter

The transcription rate of genes encoding liver-specific proteins is distinctly higher in hepatocytes as compared with other cell types(7). The transcription of several hepatic genes is activated during liver development and later modulated depending on extra-cellular stimulation(8-10). Experiments using a cDNA library from mouse liver poly(A)+ RNA that was then differentially screened with poly(A)+ RNA from liver and nonliver cells provided strong evidence that the predominant control of liver-specific gene expression resides at the level of transcription(11,12).

Clones proven to be liver-specific were picked and used as templates for hybridization with radioactive RNA newly transcribed in vitro in nuclei isolated from liver and nonliver tissues. The hybridization signals obtained with RNA synthesized with liver nuclei were at least 10 times more intense than those obtained with nuclei from other tissues. Because the cDNA clones represented an unbiased population of transcripts, the findings led to the conclusion that liver-specific gene expression is primarily a consequence of transcriptional regulation(11).

In this way, liver contains liver-high expressed genes that are mainly regulated by the many liver-enriched transcription factors binding to the 5′-flanking region. For instance, Hepatic nuclear factor 1-α (HNF1-α) is an atypical homeodomain-containing protein which was first identified based on its ability to bind to critical regulatory *cis* elements present in the 5′-flanking region of liver-specific genes, such as the albumin, β-fibrinogen, and α1-antitrypsin genes(13-15). One other example, Hepatocyte nuclear factor 4 (HNF-4) is a liver-enriched transcription factor and a member of the steroid hormone receptor superfamily. HNF-4 is required for the hepatoma-specific expression of HNF-1 alpha, another liver-enriched transcription factor, suggesting the early participation of HNF-4 in development(16).

In general, the process of liver enriched expression is controlled at the level of liver-enriched transcription factors and is mediated by differential levels of activation of genes through their promoters. Hepatocytes may selectively express transgenes linked to the promoters of the gluconeogenesis enzyme PEPCK and α-antitrypsin protease(17). PEPCK gene expression is highest in liver, but significant expression also occurs in the other gluconeogenic organ, the kidney(18). With regard to the liver-specific expression of this gene, footprinting studies were carried out using nuclear extracts prepared from rat liver, kidney, spleen, and brain and a region of the PEPCK promoter was identified that displays a distinct liver-specific footprinting pattern(19). Such liver specific expression of PEPCK promoter is controlled by cAMP. The liver-specific nature of the cAMP responsiveness of the PEPCK promoter involves the recruitment of C/EBPα to the cAMP response unit(20). Testing in chicken models showed that PEPCK promoter exhibits the potential usefulness of liver-specific gene expression(21).

Apolipoprotein E has a major role in the redistribution of cholesterol and other lipids between peripheral tissues and the liver. This promoter is expressed at high levels in liver that requires a common cis-acting regulatory domain of hepatic control region (HCR)(22). Synergistic effects of proximal and distal regulatory elements have been shown to play an important role in controlling cell-specific expression in transgenic mice, when the HCR is closely located in the promoter/enhancer of apoE(23).

The enhancer/promoter of the albumin gene is the only tissue-specific promoter that has been shown to preferentially target liver cells(23). When this promoter was delivered using a retrovirus, which is efficient only in dividing cells, *β*-galactosidase expression was restricted to hepatoma cell lines in vitro. When this retrovirus was injected via the spleen or directly into the liver, gene expression was observed only in dividing heptocytes in partially hepatectom-

ised mice, but not in nondividing hepatocytes in normal mice. These authors went on to show that the susceptibility of murine and rat hepatocellular carcinoma (HCC) cells, infected with retroviruses expressing HSV-*tk* under the control of the albumin promoter, were 100-fold more sensitive to gancyclovir than non-HCC cells. Systemic gancyclovir administration resulted in complete regression of retroviral-infected HCC cells and significant inhibition of tumor growth even when only 5% of the cells were infected with retrovirus(24). The albumin promoter has also been used to develop a retrovirus expressing the TNF-α, IL-2, and IL-3 genes(25,26), an adenovirus expressing HSV-*tk*(27,28) and a replication-competent herpes simplex virus, HSV G92A(29).

In vitro transcriptional targeting of hepatocellular carcinoma (HCC) was accomplished with α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) promoters(30). CEA is a common tumor marker, expressed in colon, hepatic, pancreatic and lung carcinomas. However, it may also be expressed in benign inflammatory conditions, albeit less abundantly. CEA sequence upstream of the CD gene can selectively sensitize HCC cells to 5-FU cytotoxicity. Multimerization of sequences between −89 and −40 resulted in copy number-related increases in both expression level and selectivity for CEA-positive cells. Two enhancer regions of CEA, −13.6 to −10.7 kb or −6.1 to −4.0 kb, were identified and support high-level and selective reporter expression in CEA-positive cell lines(31). α-fetoprotein is another relatively specific gene expressed by hepatocellular carcinoma. Its promoter has also been successfully linked to TK gene and cloned into a retroviral vector to infect and selectively kill hepatoma cells(30).

Prostate specific promoter

Prostate-specific antigen (PSA) is a well-characterized and important marker for prostate cancer(32,33). PSA is expressed predominantly in the prostate and is transcriptionally upregulated by androgens. Elements from the 5′ region of this gene can drive reporter gene expression in the PSA-producing cell line, LNCaP, but not in the nonproducing tumor cell lines, DU145 and PC-3, nor in renal or breast cancer cell lines(34). Tumor cell growth was inhibited when the PSA promoter was used to drive expression of antisense constructs targeting DNA polymerase α and topoisomerase α in prostate tumor cell lines, but not in cell lines of any other tumor(35). In addition, a replication-competent adenovirus, CN706, has been developed with a selective toxicity for PSA-positive prostate cancer cells, using minimal enhancer/promoter constructs derived from the 5′ flank of the PSA gene, to drive the E1A gene(36). Adenovirus which expresses HSV-TK of the PSA promoter was active in both androgen-dependent and independent PSA-producing prostate cancer cells in vitro, and in prostate tumors of castrated hosts. Importantly, intravenous delivery of adenoviral constructs, with either duplication of the enhancer core or insertion of tandem copies of the proximal androgen response elements, not only showed enhanced activity and inducibility but also retained tissue discrimination for human LAPC-9 tumor xenografts(37). Constructs utilising PSA promoter elements have also been developed to drive the expression of the sodium iodide transporter to concentrate radioiodine in the prostate(38). They have also been used to increase the expression of nitroreductase or CD allowing sensitisation of prostate cells to the prodrugs, CB1954(39) or 5-FC, respectively(40). PSA promoter-driven transgenes have also been delivered using an HIV-1-based lentiviral vector(41) and liposomes(42).

Human glandular kallikrein (hK2) protein is also becoming an important marker for prostate cancer. The genes for hK2 is expressed predominately in the prostate, is transcriptionally up-regulated by androgens. The expression of the hK2 gene is directly androgen regulated and specifically expressed from prostate cell(43-45). Transfection experiments with proximal hK2 promoter (-324 to +33) has been shown to contain a functional androgen-response element that is inducible in prostate cells(44). A potent far upstream 5′ regulator was characterized, which is required for high-level, androgen-regulated, and cell-specific expression of the hK2 gene. A replication-competent adenovirus was constructed, CV763, in which the E1A gene was driven by the hK2 enhancer/promoter. Specificity for prostate tumors in vivo was impressive. More recently, a recombinant adenovirus expressing EGFP under the control of a triplicate hK2 enhancer/promoter led to robust tumor-restricted EGFP expression(46).

PSMA is a membrane-bound antigen(47) and its expression is

androgen-independent(48). In addition, in contrast to PSA, PSMA is more highly expressed after androgen ablation therapy. The promoter/enhancer of PSMA is considered to be suitable for gene therapy under androgen deprivation conditions. These advantages make PSMA a useful target for advanced prostate cancer therapy, especially in combination with conventional hormonal treatment. The most active regions of the PSMA enhancer revealed a 1.6-kbp region which was active in driving prostate-specific CD expression, sensitising cells in vitro to 5-FC more than 50-fold compared to control cells(49). Furthermore, C4-2 prostate tumors grown in nude mice were eliminated by 5-FC when CD expression was induced by the PSMA promoter/enhancer(50). This type of promoter will be particularly useful for patients who have undergone androgen ablation therapy and are suffering from a relapse of the disease.

Ovarian specific promoter

Ovarian carcinoma is a leading cause of cancer death in women. Though advances in conventional therapies have been achieved, transcriptional control elements (promoters) of genes frequently upregulated or specfically expressed in tumors are less studied. However, repetitive retrovirus-like elements in the rat genome is detected in the ovarian-specific transcription units (OSTUs) in the rat ovary(51). Regulated expression of retrovirus-like elements is generally controlled by the U3 region of their 5′ long terminal repeat. The U3 region of OSTUs was cloned and is referred to as ovarian-specific promoter 1 (OSP-1) based on its ability to preferentially direct the expression of reporter genes in cell lines of ovarian lineage(52). The OSP-1 controlling the expression of T antigen and harboring in transgenic mice can selectively direct suicide gene therapy of ovarian cancer(53).

Our limited knowledge of the initiating events of ovarian cancer has restricted the development of models in which the early pathogenic events for ovarian cancer can be studied. But recently the ceruloplasmin promoter was more highly expressed in ovarian tumors and cell lines than in normal ovaries and 40% of primary ovarian carcinomas over-express ceruloplasmin. In nude mice carrying SKOV3.ip1 xenografts, the ceruloplasmin promoter demonstrated significantly higher activities in tumors compared with normal organs. Notably, the levels of promoter activity were less in the normal liver, lung, pancreatic, and ovarian cell lines(54). This promoter showed good specificity for ovarian cancer cells in vivo.

Melanoma specific promoter

The use of tissue-specific promoters was pioneered by Vile and Hart in 1993, with the use of the tyrosinase promoter to target melanocytes/melanomas. Melanin is synthesized in melanocytes, partly, as a consequence of transcriptional regulation of gene products, such as tyrosinase or tyrosinase-related protein 1, involved in this biochemical pathway. Tyrosinase, the rate-limiting enzyme in melanin synthesis, is expressed specifically in pigment-producing cells. Expression of tyrosinase is regulated by cAMP. The M-box 70-bp upstream from the TATA-box and the E-box located downstream the TATA-box, near to the initiator site, are involved in the regulation of the tyrosinase promoter activity by cAMP. Microphthalmia, a basic helix loop helix transcription factor, binds to these regulatory elements and modulates the transcriptional activity of the tyrosinase promoter. cAMP elevating agents, such as forskolin and a-MSH, lead to a strong stimulation of transcriptional activity of the tyrosinase promoter by increasing binding of microphthalmia to the M-box and to the E-box(55). Melanoma-specific expression of a transgene can be achieved by using the tyrosinase promoter, this targeted expression being further amplified by addition of the melanocyte-specific enhancer(56). They went on to demonstrate efficacy in vivo using the herpes simplex thymidine kinase (HSV-*tk*)/ gancyclovir (GCV) combination, which gave a significant tumor growth delay following intratumoural injection of the tyrosinase-driven constructs and decreased metastatic potential in mice injected with melanoma cells expressing these constructs following treatment with GCV(57). In a separate study, tissue-specific expression of IL-2, IL-4, and GM-CSF was also seen in vitro and in vivo(57). As an extension to this work, retroviruses were developed containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of HSV-*tk* or IL-2 genes(58,59). Antitumor efficacy and tissue specificity were observed after intratumoural and IV delivery using localised and metastatic melanoma models(58). Other studies have demonstrated an-

titumor efficacy using both retrovirus(60) and cationic liposomes(61) to achieve tyrosinase driven expression of cytosine deaminase (CD), which in turn catalyses the conversion of the nontoxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). Adenoviral delivery of the murine tyrosinase promoter, introduced into the E1 region, has been used to drive melanoma-specific expression of the reporter gene β-galactosidase(62). Both studies showed effective transcriptional targeting. More recently, tissue-specific elements of the tyrosinase promoter have been incorporated into more elaborate DNA constructs, that incorporate either a second tumor-specific promoter, cyclin A(63) for added specificity, or elements to help increase tyrosinase promoter expression(64). As an extension to this, both targeted delivery and transcriptional targeting of melanoma cells via the tyrosinase promoter were combined in one study. Anionic liposomes carrying a peptide ligand for targeting α-v-β 3-integrin receptors, commonly upregulated on malignant melanoma cells, were used in this instance for the transductional targeting(65). These more elaborate combined approaches will be discussed in detail later as future perspectives.

Cancer specific targeting

The division between a tissue specific and a cancer-specific promoter is a very blurred one. However, as many tissue specific promoters are confined to cancers of specific tissues, the possibility of developing a transcriptionally targeted promoter with a broader spectrum of activity is an attractive one. The development of such a system relies upon the identification of a gene/protein that is unique to a broad range of cancer types. A common problem with the use of the tissue-specific promoters which were described in the previous section, is that transgene expression can also occur in the normal tissue and whilst some tissue damage may be acceptable in certain organs, in other organs such as brain, liver, and so forth, it could be catastrophic. There is probably only one gene, telomerase, that can be genuinely classified as cancer specific and whose promoter is being used to drive the expression of transgenes selectively in a wide variety of tumor cells.

Telomerase is a ribonucleoprotein enzyme that maintains the protective structures at the ends of eukaryotic chromosomes, called telomeres. In humans, telomerase expression is repressed in most somatic tissues and telomeres shorten with each progressive cell division. In contrast, telomerase activity is a common finding in many human malignancies resulting in stabilized telomere length. It is now well documented that the level of telomerase in malignant tissue compared to normal tissue is much higher and this differential is greater than that for classical enzymatic targets such as thymidylate synthase, dihydrofolate reductase or topoisomerase II. Telomerase activation is considered to be a critical step in cancer progression because of its role in cellular immortalization. Approximately 90% of human cancers possess active telomerase, whereas normal somatic tissues have either much lower or undetectable activity(66-68). Human telomerase activity depends on the presence of both the RNA subunit (hTR) and the catalytic protein component (hTERT)(69). For both genes there is a clear differential expression between normal and malignant cells. A number of studies have used these two promoters to drive transgene expression for therapeutic benefit. First description is employed the hTERT promoter in a retroviral system and combined it with a Cre/*loxP* site-specific recombination system. It is allowed the killing of p53-negative tumor cells while sparing normal wild-type cells(70). Since then, the glioma cell line, UVW, was targeted using the hTR promoter to drive expression of the nor adrenaline transporter gene (NAT) where a 17-fold enhancement of the radionuclide 131-meta-iodobenzylguanidine uptake was reported resulting in complete sterilization of tumor spheroids(71). Gliomas were also the target for hTERT-driven expression of Fas-associated protein with death domain (FADD) or rev-caspase-6. FADD is a protein which induces apoptosis in cells regardless of Fas expression on the cell surface and rev-caspase 6 induces apoptosis independent of initiator caspases. Subcutaneously implanted human gliomas treated with this construct were significantly reduced in volume compared to control tumors(72-74). More recently, the apoptotic pathway was successfully targeted both in human tumor xenograft and syngeneic mouse UV-2237m fibrosarcomas, using Bax as a transgene, driven by the hTERT promoter(75,76). In a suicide gene therapy approach, hTERT was also used to drive expression of HSV-*tk* in an osteosarcoma xenograft model following intratumoural injection.

Targeted delivery using this approach avoided the liver toxicity observed using a CMV driven construct following GCV administration. Keith's group(77) tested hTR and hTERT in a panel of 10 cell lines consisting of lung, colon, ovarian, bladder, and cervical origin, demonstrating the truly "cancer specific" nature of these promoters. The hTR promoter appeared to have the superior activity in the telomerase-positive cell lines and when used to drive the nitroreductase gene to increase the activation of the prodrug, CB1954, in two xenograft models, it showed even better antitumor effects (up to 97% reduction in tumor volume) than a CMV-driven construct(79). These promoters clearly have a real potential in targeting a wide range of different tumor types.

Efforts for manufacturing the activity of weakly tissue specific promoter

The transcriptional activity of the tissue-specific elements used as promoters for gene therapy is often too weak to generate adequate concentrations of transgene product in target cells. Given that in vivo gene delivery to the tumor cells might be limited, a concern for employing weak specific promoters is that therapeutic efficacy might decline. The dilemma is how to achieve high levels of transgene expression without compromising specificity.

Simple manipulations of known regulatory elements, such as removal of a negative and inert regulatory sequence or multimerization of positive elements, can promote synergistic and cooperative interactions of activators to enhance transcription. For example, the activity of native PSA promoter and enhancer (PSE) can be augmented by modifying the androgen receptor (AR) elements, which serve key activating functions for the PSA gene(78,79). By insertion of four tandem copies of the synthetic androgen-responsive element, or by duplication of a 400-base pair enhancer core element, a nearly 20-fold enhancement of activity over the parental PSE was achieved(80). Similar approaches have been successful in improving the activity of the tyrosinase promoter(81) and the CEA promoter(82). An interesting and more extreme approach would be to generate a complete synthetic promoter by multimerization and shuffling of known regulatory elements, then selecting the most active and properly regulated construct. This approach has been applied to the chicken skeletal α-actin promoter to achieve muscle-specific expression that exceeds the level of the CMV promoter(83). Other useful strategy is to generate hybrid promoter, MCK/CMV, derived by the enhancer element E1 from the tissue specific muscle creatine kinase (MCK) promoter, which has been suggested to be restricted to differentiated, multinucleated myofibers, in combination with the CMV promoter/enhancer(84). The use of hybrid promoters which combine tissue specificity and strong expression have been demonstrated to be a promising tool for gene therapy vectors(85-87).

The second strategy has frequently been employed to amplify weak promoter activity in a two-tiered manner. The engineering of cell-specific enhancers to improve potency and the development of two-step transcriptional activation (TSTA) approaches have significantly improved the efficacy of transgene expression. The specific promoter directed the potent transcription activator, GAL4-VP16, which in turn acted upon a second GAL-4-responsive reporter or therapeutic gene. This TSTA approach, based on the original 'enhancer trap' methodology to study gene expression in *Drosophila melanogaster* development, can boost the activity of the PSA promoter over a range of up to 1000-fold. Optimal TSTA constructs displayed activity levels significantly higher than those of the CMV promoter, while maintaining prostate cell specificity and androgen responsiveness(88,89). An alternative strategy using a GAL4/VP16 fusion protein to enhance the weak tumor-specific CEA promoter achieved similar amplification of the reporter genes *LacZ* or *GV16*(90). This concept has been further developed by using a tissue-specific promoter to drive expression of GAL4 derivatives fused with up-to-4 VP16 activation domains. By incorporating up-to-5 GAL4 binding sites upstream of the reporter gene (firefly luciferase) 800-fold amplification was achieved(90).

In another approach, a cancer-specific promoter that controls the expression of Cre site-specific recombinase(91), the Cre/*loxP* system, offers a different approach to the enhancement of tissue-specific promoter activity, which activates the

reporter or therapeutic gene expression in a second step. Target cells are transfected with a plasmid incorporating the therapeutic transgene separated from a strong constitutive promoter by a "stop" cassette enclosed by two *loxP* sites. Cotransfection with a site-specific Cre gene driven by the tissue-specific promoter generates Cre, which excises the stop cassette, bringing the therapeutic transgene under the control of the constitutive promoter(92). The cell-specific, Cre-dependent activation of transgene expression was demonstrated in CEA-targeted systems(93), in thyroid carcinoma-targeted therapy(94), in a growth hormone promoter-mediated strategy targeting pituitary tumor(95), and in AFP promoter-based therapy for liver tumor. The specificity of this system is maintained when the Cre and the loxP component are inserted in two separate vectors. However, the activation of this system requires co-delivery of two vectors into the same cell, which could be inefficient in vivo(96).

Strategies for increasing specificity

Using the ribozyme expressed from tissue specific promoter

Tissue-specific expression of the target-specific ribozyme can be very useful approach to the specific treatment of malignant disease. For example, the apolipoprotein B mRNA-specific hammerhead ribozyme (RB15) driven by a liver-specific transthyretin (TTR) promoter(97) using the AAV2 serotype virus vector (rAAV2-TTR-RB15). As the overproduction of apolipoprotein B (apoB) is positively associated with premature coronary artery diseases, apoB mRNA can be a potential target for gene directed therapy for liver cancer. ApoB mRNA-specific hammerhead ribozyme that mediated by rAAV efficiently cleaves and decreases apoB mRNA by 80% in mouse liver and attenuates the hyperlipidemic condition(98). An alternative strategy is to prevent *mdr1* mRNA expression using anti-sense RNA or ribozyme(99,100) derived by a retroviral vector coupled to the carcinoembryonic-antigen (CEA) promoter. Because P-glycoprotein (Pgp)-conferred multidrug resistance (MDR) is expressed in cancer and in normal colon tissues and has important physiological function, reverse MDR by the hammerhead ribozyme in malignant tissue was selectively expressed without disrupting the function of normal colonocytes. An anti-mdr1 hammerhead ribozyme coupled to a CEA promtoer can establish the selective killing in CEA-expressing human colon-adenocarcinoma cells(101).

Expansion of the specificity of gene delivery

Specificity can be obtained in two principal ways. One option is 'physical targeting' which relies on the spatial focus of physical methods such as gene gun, hydrodynamic delivery, ultrasound, magnetofection, electroporation, hypothermia, or photodynamic therapy. A directed mechanical force, magnetic or electrical field, temperature or light can be exploited for specific localization. Importantly, the efficiency of some of the methods is already very high (for example, hydrodynamic delivery of genes to rodent livers), which has triggered their broad use in pharmacological in vivo models. Applications of physical targeting strategies for cancer therapy depend on either direct local action at the treated tumor site or a distant systemic action of locally applied (e.g. skin, muscle) genes with anti-angiogenetic or anti-tumor immunostimulatory properties. For instance, in vivo eletroporation is a technique that applies short-duration electric field pulses to various tissue after local administration of genes. The cell membranes is destavilized by the electrical stimulus, allowing passage of the gene directly into the cell cytoplasm, avoiding endocytotic pathways into degradative vesicles, and enhancing transgene expression by two or three orders of magnitude(102,103). Anti-melanoma immune responses were demonstrated by combined electroporation of DNA encoding gp100 and TRP-2 antigens(104). Intramuscular plasmid electrotransfer of secretable forms of antiangiogenic factors was sufficient to inhibit tumor growth in several models(105). In tumors, injection of DNA followed by local electroporation has also been shown to enhance gene transfer. Therapeutic effects were observed upon intratumoral electroporation of IL-12 in the B16F10 melanoma model(106) or with the HSV thymidine kinase gene followed by systemic ganciclovir treatment in the C26 colon carcinoma mouse model(107).

The second option capitalizes on special biological and pharmacological characteristics of the tumor target. By taking advantage of the hyperpermeability and deorganization of tumor vasculature and the inadequate lymphatic drainage of solid tumors, sufficiently long circulating particles accumulate in tumor tissues. Exploiting the enhanced permeability and retention (EPR) effect of circulating particles has also been termed a 'passive targeting' strategy. Alternatively, active targeting can be achieved when specific ligands are coupled to the carrier systems which bind to cell surface molecules overexpressed on the target tumor tissue. Target molecules can be specific receptors on tumor cells(108,109) or the tumor vasculature(110). Active targeting process which can lead to enhanced internalization of the particle by receptor-mediated endocytosis are particularly attractive.

Conclusions

The ultimate goal of all currently used cancer therapy is to target damaging events to tumor cells while sparing normal tissues sufficiently that they can recover to an adequate functional levels. Ideally, expression of therapeutic genes driven by cancer-specific promoters would only target tumors resulting in minimal toxicity to normal tissues. However, even these therapeutic effects of minimal toxicity are deleterious to normal tissues, and hence cell-type specific targeting is essential. Consequently the use of tumor and tissue-specific transcription regulatory elements may both restrict transgene expression to malignant tissues and increase transgene expression and persistence in vivo. On this account, conventional therapies rely on one, or at the most two, targeting characteristics such as spatial deposition, biochemical pathway specificity, or cellular proliferation. However, there is no theoretical limit to the number of elements that can be incorporated into a gene therapy strategy to confer tumour specificity. There is also the potential in gene therapy for controlling temporal expression of transgene products in tumors much more precisely than the concentration of conventional anticancer agents.

The first opportunity for targeting specificity is transcriptional targeting. It is unlikely for most cancers that any one promoter will offer the ideal combination of interrelated characteristics: high level specificity to endogenous or exogenous factors, silence in the absence of these factors, and sufficient strength to induce the therapeutic transgene many fold. In any event, the use of a single promoter/transgene combination fails to exploit one of the main advantages of gene therapy: the ability to custom design, even within a single plasmid, a combination of diverse elements that confer exquisite spatial and temporal specificity for an individual tumour type. It may even be possible using gene/protein array technology to tailor this specificity to an individual patient's tumour. It could be envisaged that promoters specific to the tumour tissue type could be combined with factors inducible by the tumour microenvironment and/or exogenous control elements (eg, radiation). This has already been successfully achieved in vivo by combining cell cycle and tyrosinase promoters(111), HREs and KDR/E- selectin promoters(112), and HREs and the AFP promoter(113). Furthermore, these could be combined with elements incorporating strong promoters/enhancers in positive feedback loops giving progressively stronger and more specific transgene expression(114,115) or by using a Cre/*loxP* system(116). The packaging of such complex constructs, ideally within a single construct, will require a more thorough understanding of the control domains from individual promoters such that multiple copies of the elements that confer tumour specificity can be incorporated while eliminating those sequences that permit expression in nontarget tissues.

A vital issue when considering the clinical application of any gene therapy protocol is the basal expression of the transgene. Many of the papers discussed in this review have failed to adequately assess basal-level expression in other tissues following systemic delivery in an appropriate in vivo model. Highly sensitive systems now exist for assessing promoter leakiness in vitro(117) and recent developments in imaging technology have permitted whole animal detection of fluorescent reporter gene products in small rodents(118,119). Although this technology is theoretically applicable in man, considerable caution must be exercised in extrapolating from animal data. It is likely in early trials that transgene activation by a given promoter combination will need to be assessed using a nontoxic reporter gene, particularly where tis-

sue-specific promoters are to be used. This use of a surrogate marker is an additional and important advantage of gene therapy over conventional chemotherapy that can be applied even at the level of the individual patient.

Another opportunity for targeting specificity is at the level of vector delivery. Direct injection systems have been important in providing the proof of principle for several gene therapy strategies, but the number of accessible sites will always be limited and perhaps more importantly the crucial problem of disseminated disease cannot be addressed.

Using the technologies described in this review, there is considerable optimism that it will eventually be possible to restrict therapeutic gene expression tightly to tumor cells. This will provide one part of the overall package required for delivery of effective genetic therapies for cancer. Transcriptional targeting of gene expression is, therefore, a central weapon in the genetic oncologist's arsenal which will reduce toxicity to normal tissues while selectively and efficiently eradicating tumor cells, clearing the way for gentler and more effective treatments for cancer patients.

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