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Review

Targeting gene therapy vectors to CNS malignancies

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Gene therapy offers significant advantages to the field of oncology with the addition of specifically and uniquely engineered mechanisms of halting malignant proliferation through cytotoxicity or reproductive arrest. To confer a true benefit to the therapeutic ratio (the relative toxicity to tumor compared to normal tissue) a vector or the transgene it carries must selectively affect or access tumor cells. Beyond the selective toxicities of many transgene products, which frequently parallel that of contemporary chemotherapeutic agents, lies the potential utility of targeting the vector. This review presents an overview of current and potential methods for designing vectors targeted to CNS malignancies through selective delivery, cell entry, transport or transcriptional regulation. The topic of delivery encompasses physical and pharmaceutic means of increasing the relative exposure of tumors to vector. Cell entry based methodologies are founded on increasing relative uptake of vector through the chemical or recombinant addition of ligand and antibody domains which selectively bind receptors expressed on target cells. Targeted transport involves the potential for using cells to selectively carry vectors or transgenes into tumors. Finally, promoter and enhancer systems are discussed which have potential for selectivity activating transcription to produce targeted transgene expression or vector propagation.

Keywords: CNS malignancies; brain tumor; vector; targeting; gene therapy; glioma; glioblastoma

Introduction

Therapy for CNS malignancies, in particular intracranial malignant gliomas, currently relies on the three modalities surgery, radiation and chemotherapy. With modern treatment paradigms the median survival time of patients with the most malignant glioma, glioblastoma multiforme (GBM), has increased from several months to over a year in selected series (Walker *et al*, 1978; Kristiansen *et al*, 1981; Gutin *et al*, 1991). Survival in patients with other intracranial primary neoplasms and metastatic disease has likewise increased. Although several of these diseases now have significant cure rates, the majority are eventually lethal in a large proportion of patients. Each therapeutic modality has its own mechanism and profile of selective elimination of tumor cells as opposed to normal cells, and thus provides a unique contribution to the therapeutic ratio. To a large degree, therapies are combined such that additive or synergistic toxicity to tumor cells is maximized, while toxicity to normal tissue is minimized. Clinical tumor burdens may consist of 10^9-10^{12} cells, of which a therapeutic regimen must eliminate all competent clonogens to result in a cure. This goal is frequently not achieved because each modality first reaches a point at which its toxicity to vital normal tissue is limiting and overlaps with other existing agents.

Over the past 10 years a number of synthetic and viral vectors have been developed to deliver transgenes which have potential therapeutic applications in treating malignancies of the CNS. The advantage of gene therapy lies in the potential for additional, precisely designed, selective toxicity for tumor cells. In conjunction with current therapies, gene therapy may allow for selective elimination of remaining tumor cells, resulting in prolongation of survival or cure. The source of selectivity can range from preferential toxicity of the transgene product

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to targeting of vector activity to the tumor or affected tissue. The principal focus of this review, vector targeting, can be accomplished by several means including selective vector delivery, cell entry (receptor targeting), intracellular transport or transcriptional activation (promoter targeting). Although high grade gliomas are referenced primarily in this article, many of the techniques are applicable to a broad range of malignancies in the CNS and other sites.

Targeted delivery

The most direct means of targeting a therapeutic agent is through the means and location of delivery. A straightforward method is surgical placement into the tumor mass or resection cavity. Because the two most common adult intracranial malignancies, exogenous metastases and gliomas, frequently involve the brain in a diffuse and microscopic manner, intrathecal and intravascular administration have been investigated as more pervasive means of delivery. Intravascular delivery is affected by the unique characteristics of the normal CNS vasculature and tumor neovasculature. The former composes the relatively impermeable blood brain barrier (BBB), the latter is more permeable but associated with unusual fluid dynamics, and both react differentially to various manipulations. Attempts have been made to exploit these differential characteristics through vector design and pharmacologic manipulation.

Initial protocols with most vectors have employed stereotactic intratumoral injection of virus or virus-producing (packaging) cells (Chiocca *et al*, 1994; Oldfield *et al*, 1993; Kramm *et al*, 1995). This can provide relatively efficient gene delivery to focal regions in circumscribed solitary tumors, but does not address local invasion, distant migration or diffuse seeding by tumor cells (Berens *et al*, 1990). Intrathecal delivery of vectors or packaging cells is a technique that provides more extensive exposure for tumor foci growing in or around CSF spaces, and can facilitate transduction of a higher percentage of tumor cells under such conditions (Kramm *et al*, 1996; Culver *et al*, 1992). Using an HSV-derived vector, which propagates on site in dividing tumor cells, this method has produced an even distribution of transgene delivery to tumor throughout the CSF and within the brain tissue adjacent to the ventricular space (Kramm *et al*, 1996).

Since almost all viable tissue requires vascular support, intravascular administration appears to present the greatest potential for delivering a vector to the largest proportion of tissue at risk for involvement by a diffuse or multifocal tumor. Retroviral vector administered through the portal vein has already been shown to preferentially transduce tumor deposits compared to normal liver (Hurford *et al*, 1995). This effect may result from the intrinsically high permeability of tumor induced neovasculature, mechanical disruption of vascular integrity by tumor or the higher mitotic rate of tumor cells. Albeit dependent on tumor location and individual vascular anatomy, intracarotid administration is expected to provide more intracranial lesions with the highest relative exposure to vector.

The existence of a tight blood-brain-barrier (BBB) throughout the normal brain provides an additional advantage in terms of vector selectivity for CNS neoplasms. The varying permeability found throughout the blood-tumor-barrier (BTB) in the majority of brain tumors (Bergstrom *et al*, 1983), however, may restrict maximal penetration by vector. The BTB can be further manipulated to increase permeability to small particles such as viruses. Several studies have focused on transient osmotic disruption of tight junctions between endothelial cells, and this technique has been well characterized in animal models and in humans as an enhancer of chemotherapeutic drug and vector delivery to brain tumors (Doran *et al*, 1995; Neuwelt and Hill, 1987; E4">, 1995). With mannitol disruption of the BBB, however, delivery and uptake of therapeutic agents is less specific to the tumor and there can be toxic side effects (Zunkeller *et al*, 1996).

Recently, it has been demonstrated that BTB disruption by low-dose bradykinin (BK) can facilitate selective uptake of intra-arterially administered HSV vectors to single or multiple tumor foci in the rodent brain, with essentially no infection of normal neurons and glia (Rainov *et al*, 1995. BK, a nonapeptide hormone that interacts with specific B1 and B2 receptors on endothelial cells opening tight junctions and enhancing endocytosis (Elliot *et al*, 1996; Bartus *et al*, 1996), selectively increases the permeability of tumor capillaries when infused intra-arterially at low doses (Inamura *et al*, 1994). Rainov *et al* (1995) demonstrated that transgene expression after intra-arterial BK infusion and HSV-1 vector administration is particularly intense in the periphery of the tumor, a zone with distinct biological and biomechanical properties such as high mitotic rates, angiogenesis, parenchymal invasion and low interstitial pressure (Boucher *et al*, 1996). Up to 25% of tumor cells in this region express transgene proteins after BK/HSV-1 administration, as compared to less than 0.1% of cells in normal brain tissue (Rainov *et al*, 1995). In an attempt to replace BK with a new long-acting derivative, the synthetic octapeptide RMP-7 (Alkermes Inc.), which is approved for human studies and acts more selectively on endothelial cells (Elliot *et al*, 1996), is currently being tested. Initial studies have shown it to be equivalent to BK in selectively increasing HSV-1 transduction (Barnett *et al*, manuscript in preparation).

Receptor targeting

Most viruses, which most vectors are or resemble, use viral surface proteins that bind to specific cell surface molecules (receptors) as the primary means of initiating cellular attachment. Expression of the receptors on a single or limited range of cell types produces the tissue tropism seen with many viruses. This effect is frequently a major determinant in the disease syndrome produced. A separate domain of the binding protein, an associated protein or a completely unrelated protein usually provides a subsequent and usually less specific membrane fusion or penetration function. Many of the commonly used viral vectors actually infect a relatively broad spectrum of host cells, and the non-viral vectors have almost no intrinsic selectivity. Since the therapeutic target in a gene therapy strategy is frequently a single cell type or subset, particularly in an oncologic setting, significant interest exists in increasing specificity of infectivity by adding or altering receptor binding moieties. This has been attempted by attaching or conjugating various receptor ligands and specific antibodies, as well as by recombinant modification of viral surface molecules with binding domains from ligands or antibodies. Adding surface conjugates or altering viral surface proteins may also serve to obscure native binding functions and truncate the vector's ability to effect endogenous, unwanted tissue tropisms or non-specific infectivity.

A number of nonviral vectors have been targeted by introducing specific ligands into DNA-polylysine complexes. Folate (Gottschalk *et al*, 1994), epidermal growth factor (EGF) (Cristiano *et al*, 1996) and transferrin (Wagner *et al*, 1992) have been used to target tumor cells or specific tissues which overexpress the corresponding receptor. Alternatively, streptavidin has been incorporated into the complexes, allowing the attachment of varying biotinylated ligands and antibodies (Schwarzenberger *et al*, 1996). Defective or inactivated adenovirus is frequently included as an endosomal lysis agent, thereby increasing transfection efficiency (Gao *et al*, 1993). Targeting ligands and antibodies have also been added to liposomes. Transferrin, for example, has been solubilized into liposomes and shown to target them to tumor cells which overexpress transferrin receptor (TfR) (Cheng, 1996). Antibodies against TfR (Huwyler *et al*, 1996) and the polyoma-virus-induced tumor associated antigen (Emanuel *et al*, 1996) have also been used to effectively target vectors. Specific spatial targeting of viral or non-viral vectors could also be accomplished through conjugation or adsorption of ferrofluids (biocompatible iron-oxide compounds), which have previously demonstrated efficacy in targeting pharmaceuticals using magnetic fields (Lubbe *et al*, 1996).

With current recombinant technology, the logical extension of this strategy is to alter the peptide sequences of the viral surface proteins themselves. The Moloney murine leukemia virus (MMLV) gp70 envelope protein, which mediates binding to ubiquitously expressed amino acid transport proteins (Albritton *et al*, 1995), has been modified in a variety of ways and expressed *in trans* in packaging cell lines (pseudotyping). Kasahara *et al*, inserted the receptor-binding domain of erythropoietin and achieved increased transduction of erythropoietin receptor-bearing human cells, including erythroid and erythroleukemia cell lines, and decreased transduction of cell lines not expressing erythropoietin receptors (Kasahara *et al*, 1994). A range of tumor selective ligand epitopes have been utilized in similar systems (Table 1). Modifications have also been introduced into the fiber protein of adenovirus. For example, Michael *et al*, have inserted a binding epitope from gastrin releasing peptide (Michael *et al*, 1995). Wickham *et al*, inserted a FLAG peptide then used bispecific anti-FLAG/anti-α_v integrin monoclonal antibodies (MAb) to target human venule endothelial cells and intestinal smooth muscle cells bearing α_v integrin (Wickham *et al*, 1996).

An important derivation of this strategy has been the recombinant insertion of specific antibody binding domains, with the heavy and light chain variable regions combined in a single chain fragment variable (scFv), into viral surface proteins. Germinal work was done by Russel *et al*, placing an scFv against 4-hydroxy-5-iodo-3-nitrophenacetyl capronate (NIP) (Russel *et al*, 1995) into the MMLV gp70 envelope protein. Antibody fusion proteins have subsequently been created to target MMLV to cells bearing MHC class I (Marin *et al*, 1996) and LDL receptors (Somia *et al*, 1995), and spleen

Table 1 Tumor targeting ligands recombined into retrovirus envelope

Ligand	*Targeted tumor type (receptor)*	*Reference*
Erythropoietin	Erythroleukemia (EPO-R)	Kasahara *et al*, 1994
Epidermal growth factor (EGF)	Epithelial malignancies (EGFR)	Cosset *et al*, 1995
Heregulin	Breast (ErbB)	Schnierle *et al*, 1996b Han *et al*, 1995
Neurotensin	Colon (neurotensin receptor)	Schnierle and Groner, 1996a
Urokinase-type plasminogen activator	Invasive tumors (uPA-R)	Schnierle and Groner, 1996a

necrosis virus (SNV) to cells bearing the hapten DNP (Chu *et al*, 1994). Caution must be exercised with such methodologies in that recombinant viral proteins are frequently improperly processed and packaged, resulting in functionally inactive forms (Gray and Roth, 1993; Schnierle *et al*, 1996b).

A number of protein moieties have been characterized that may have particular potential in targeting vectors specifically to the CNS or CNS malignancies via ligand-receptor interactions. The malignancy-associated extracellular matrix protein, tenascin, for which $\alpha_8\beta_1$ integrin is a receptor, is expressed by a large proportion of glioblastomas (Ventimiglia *et al*, 1992; Higuchi *et al*, 1993). MAb to tenascin have been administered stereotactically and intravascularly as ^{131}I-immunoradiotherapeutic conjugates, which localized to tumors at ratios ranging up to 200 : 1 compared to normal brain, and resulted in prolonged survival in rat models and radiographic responses in humans (Zalutsky *et al*, 1989; Lee *et al*, 1988; Bigner *et al*, 1995). Vitronectin, a matrix adhesion protein whose receptors include $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, is preferentialy expressed by astrocytomas where they actively invade brain parenchyma, and levels of expression correlate with degree of malignancy (Gladson *et al*, 1991, 1995). Brain-enriched hyaluronan-binding protein (BEHAB) is another glioma-associated matrix protein which has recently been characterized by Jaworski and Hockfield (1996). Expressed transiently during embryonic gliagenesis and migration, BEHAB is absent in the adult brain except in glia-derived tumors, in which levels of expression correlate with the capacity for parenchymal infiltration.

When considering receptor targeting for treating CNS malignancies, questions arise as to which anatomic structures other than the tumor, such as the tumor neovasculature, brain parenchyma or CNS vascular endothelium, should be primarily targeted. The diffuse nature of many CNS neoplasms and the existence of non-neovascularized microscopic tumor deposits suggests that widespread delivery to the latter two structures might be advantageous for vectors selectively toxic to neoplastic cells. The vascular endothelium of tumor or brain may provide a functional, pervasive and accessible target. A non-cytocidal replication-competent vector could primarily infect endothelium then delivery progeny to the parenchyma behind. Replication-conditional vectors that selectively propagate in and kill mitotic cells, may do the same in rapidly proliferating neovascular endothelium and tumor. Yuan *et al*, have also shown that liposomes of 400 nm in diameter can cross gaps in neovascular endothelium (Yuan *et al*, 1995). The largest of the currently used viral vectors, HSV-1, is 180 nm in diameter and, as discussed above, has been shown to cross into tumors. Thus vector passing through the blood stream could be retained by vascular endothelial receptors and diffuse across into the tumor, or even have direct exposure to receptors expressed on tumor cells themselves. Although viral vectors can access the tumor parenchyma from the vascular space, the hematologic and interstitial flow and pressure dynamics of tumors may variably curtail vector diffusion (Netti *et al*, 1995). Endothelial cells are also known to transcytose the ligands for specified receptors, including transferrin (Fishman *et al*, 1987), insulin (Ben-Shacher *et al*, 1988) and low-density lipoprotein (Urien *et al*, 1987), across the BBB, and this function has already been utilized for transport of pharmaceuticals (Frieden, 1993a). For vectors targeted to such receptors, however, there is a risk of fusion with and primary transduction of the endothelial cells, rather than passage across them. Such apparent barriers may actually not abrogate therapeutic efficacy. In addition to providing a potential source of progeny vector to tumor cells, transduction of tumor vascular endothelium that results in endothelial cell toxicity would be expected to have some therapeutic effect though vascular deprivation of the tumor, as protocols with a number of anti-neovasculature agents, such as angiogenesis inhibitors, have indicated (Yanase *et al*, 1993). The phenotypic similarities of neovasculature among different tumor histologies may provide additional advantages in extending the spectrum of tumors targeted by a single vector. Vascular endothelial growth factor receptors (VEGFR) present a potential target given their relatively selective expression on tumor neovasculature and critical role in angioneogenesis (Plate *et al*, 1994; Hatva *et al*, 1995). Transferrin receptors (TfR) present another useful target, since they are preferentially expressed on brain and probably neovascular capillary endothelium (Jefferies *et al*, 1984; Dore-Duffy *et al*, 1994), as well as on a number of tumors originating in the CNS, including medulloblastomas, neuroblastomas and glioblastomas (Jefferies *et al*, 1984; Martell *et al*, 1993). TfR can be targeted using either transferrin or anti-TfR monoclonal antibodies. The latter appear preferable due to lack of competitive inhibition of binding by normally high levels of endogenous circulating transferrin. Various pharmaceuticals have been targeted to the CNS and tumors using anti-TfR antibody conjugates (Ito *et al*, 1991; Frieden *et al*, 1991, 1993b). Toxin conjugates have eradicated glioma implants in animal models when given through intravascular or intratumoral routes (Laske *et al*, 1994; Ito *et al*, 1991). Recent reports have documented increased transfection of HeLa cells by adding transferrin to liposomes (Cheng, 1996) and increased delivery of daunomycin to the brain in rats using

liposomes conjugated to the Ox26 anti-TfR Mab (Huwyler *et al*, 1996). We have conjugated Ox26 to an HSV-1 vector and demonstrated increased transduction of lacZ into TfR-upregulated myoblast and gliosarcoma cell lines (Spear *et al*, 1997).

Phage display may provide an additional source of recombinant peptide sequences for targeting. The technique initially utilized for isolating small peptides of specified affinity by Parmley and Smith (1989) and Ladner and Guterman (1990) in the early 1990s. Nearly random oligonucleotide sequences are inserted into the filament binding protein of an *E. coli* filamentous phage (frequently protein III of the M13 phage) to generate a library of phage expressing approximately 10^7-10^9 different peptides. Phage expressing a peptide sequence having high affinity for a specific molecule or tissue can then be selected out for expansion by *in vitro* binding and elution (Scott and Smith, 1990) or *in vivo* hematogenous administration and organ specific recovery (Pasqualini and Ruoslahti, 1996). The DNA sequences coding for the binding peptides are subsequently recovered from the phage genome. Peptides that target a number of molecules and tissues, including the brain, have been reported (O'Neil *et al*, 1995; Pasqualini and Ruoslahti, 1996).

Targeted cellular transport (carrier cells)

Viral vectors are typically generated in culture using packaging cells (retrovirus) or permissive cells (HSV and adenovirus). Depending on the type of vector, the viral genome may be introduced into these cells in intact or in segments. Progeny virus is produced and released either with or without cytopathic effect on the host cell, again depending on the specific vector and system. The fact that translated viral products derived from physically separate DNA sequences placed in the same host cell *in trans* can be incorporated into the same progeny virion, has been used to express many of the recombinant binding proteins discussed above. Packaging cells producing retroviral vectors have previously been placed into the brain stereotactically (Chiocca *et al*, 1994; Oldfield *et al*, 1993 rep Short *et al*, 1990). Although most commonly used vector producing cells have little or no migratory potential or specific tropisms, other cell types with these properties could be used to transport vector production to and within CNS tumors in a targeted manner.

Lymphocytes comprise one of the most promosing cell populations for carrier-mediated delivery. Lymphocytes circulate throughout the vascular system and infiltrate tissues, and selected lymphocyte populations appear to home to tumors and the CNS. In vitro-sensitized (IVS) lymphoid cells and lymphokine-activated killer (LAK) cells have been isolated from the peripheral hematolymphoid system and expanded in culture using IL-2, with or without tumor cell co-culture. When infused intravenously, IVS and LAK cells have been reported to attack and eliminate pulmonary and hepatic micrometastases in murine models (Mule *et al*, 1984; Papa *et al*, 1986; Lafreniere and Rosenberg, 1985; Shu *et al*, 1987), as well as metastatic tumors in a variety of sites in humans (Schoof *et al*, 1988; Negrier et al, 1989). Tumor infiltrating lymphocytes (TIL), isolated from melanoma, renal cell carcinoma, lung carcinoma, breast carcinoma, colon carcinoma and other malignancies, can home to, infiltrate and elicit complete responses in metastatic tumor deposits when injected intravenously (Itoh *et al*, 1986; Kurnick *et al*, 1986; Rabinowich *et al*, 1987; Miescher *et al*, 1987). Specifically, ^{111}In-labeled TIL uptake in extra-cranial tumors 3–40 times higher than normal tissue has been seen, with up to 4.5×10^7 TIL estimated to infiltrate each gram of tumor (Fisher *et al*, 1989; Griffith *et al*, 1989). The ability of TIL to selectively home, however, has recently been questioned by one study that failed to show selective tumor uptake of NeoR marked TIL (Nerrouche *et al*, 1995). Further, the CNS presents a potentially more complicated target given its supposed, albeit disputed, status of immune privilege (Weller *et al*, 1995, 1996). Although trials using locally administered LAK to treat gliomas have indeed not demonstrated therapeutic efficacy (Weller *et al*, 1995), a cytotoxic T-Cell response, as well as functional LAK and TIL have been observed in a significant proportion of gliomas (Paine *et al*, 1986; Kuppner *et al*, 1988; Yoshida *et al*, 1989). Trafficking of systemically administered TIL to gliomas, albeit of low efficiency, has also been reported in murine tumor models (Saris *et al*, 1992). Recent reports from Inoue *et al*, have demonstrated infiltration and elimination of murine intracranial fibrosarcoma implants by a subset of intravenously-administered, bacterial superantigen-activated T cells (Inoue *et al*, 1996). Specific T cell populations have also been characterized that home to and infiltrate the CNS and PNS (peripheral nervous system) in experimental allergic encephalomyelitis (EAE) (Linington *et al*, 1993) and experimental allergic neuritis (EAN) (Kramer *et al*, 1995) animal models, respectively.

TIL have already been successfully used to transport transgenes to tumors. Initial studies demonstrated infiltration of tumors with TIL which had been transduced *ex vivo* with the NeoR marker gene using a MMLV retroviral vector (Rosenberg *et al*, 1990). MMLV-transduced TIL have further been used to transport tumor necrosis factor alpha (TNFa) expression into tumors with therapeutic intent (Hwu *et al*, 1993, 1997). T cells have also been transduced *in vitro* and used to deliver nerve growth factor (NGF) production to the peripheral nervous system in the aforementioned EAN model

(Kramer *et al*, 1995). The problem still remains that many viruses, including adenovirus and HSV, can be rapidly cytotoxic to their host cells. Also, a hematogenously disseminated carrier cell that constituitively produces vector will result in non-specific systemic exposure to vector until it is sequestered in its target tissue. Constituitive expression of viral antigens may also result in immune mediated elimination of the carrier cell.

Several of these obstacles could be circumvented by stereotactically implanting cells that would migrate away from the primary tumor site, ideally following the paths of infiltrating malignant cells. Several non-malignant, migratory cells lines have been described. Rat endothelial cells, immortalized by transfection with E1A, have been shown to migrate across subcutaneous glioma implants and incorporate into the tumor neovasculature (Lal *et al*, 1994). The murine neural progenitor cell line, C17-2, is highly migratory in the CNS (Snyder *et al*, 1992) and appears to distribute throughout experimental tumors in the rat brain (Aboody-Guterman *et al*, 1996).

Further potential exists for engineering lymphocyte or non-lymphocyte cell surface receptors to create affinities that will target them to specified cell types or structures. A number of receptors and ligands have been implicated in homing, diapedesis and activation of lymphocytes and metastatic cells in the CNS, including L-selectin (Huang *et al*, 1991), CD11a (LFA-1), ICAM-1 (Greenwood *et al*, 1995), CD44, hyaluronate (Aho *et al*, 1994), VCAM-1 and $\alpha 4$ integrin (Weller *et al*, 1996; Engelhardt *et al*, 1995). Myelin protein P2 and myelin oligodendrocyte glycoprotein are thought to be the lymphocyte homing targets in the aforementioned EAN (Kramer *et al*, 1995) and EAE (Linnington *et al*, 1993) models. Bombesin, a neuropeptide growth factor, is a known chemoattractant for monocytes and small cell lung carcinoma cells that is theorized to play a role in the development of brain metastases, and thus potential exists for use of its receptor (Ruff *et al*, 1985). Tumor-specific T cell receptors (TCR), specifically those recognizing the MART-1 melanoma antigen, have been cloned and expressed in T-cell lines to direct activity against melanoma cells (Cole *et al*, 1995). Antigen binding regions from antibodies have also been expressed on lymphocytes and used to target them to tumor cells (Weijtens *et al*, 1995). scFv fragment from MAb have been placed into TCR (Gross *et al*, 1989), and T cells transduced with an anti-folate binding protein scFv-TCR chimera expression cassette have been used to treat metastatic ovarian tumors in a murine model (Hwu *et al*, 1995). Many of the antigens, receptors and ligands listed in previous sections are potential targets for directing engineered carrier cells to gliomas and other CNS tumors. This strategy may be complicated by the likelihood that not a single receptor, but specific combinations of receptors and activated signaling pathways are involved in lymphocyte trafficking.

Transcription and replication targeting

In the case of a broadly disseminated vector or carrier cell, selective activation of transcription and/or replication can provide a means of limiting non-target tissue exposure to vector or transgene product. Many viruses intrinsically prefer to function and propagate in mitotic cells, such as tumor cells, which provides some native targeting. This differential towards tumors can be enhanced by deleting genes which are critical for replication in non-dividing cells or modifying transcriptional regulation through selective promoters. Targeting through promoter sequences has been founded in restricting therapeutic transgene expression to target tissue. A broad range of useful promoter and enhancer sequences have thus far been characterized, including those whose function is dependent on cell type, cell cycle status and external stimuli. These allow for great diversity and specificity in tailoring a vector to its intended use.

Most currently used viral vectors preferentially replicate, activate transcription and/or integrate in rapidly dividing cells. This creates marked differential in the CNS where most normal glia and neurons are post-mitotic. In many viruses the selectivity for dividing cells can be enhanced by relatively simple methods. HSV-1, for example, is made nearly replication-conditional to cell division and has demonstrated selective toxicity to tumors, with the disruption of ribonucleotide reductase (Goldstein *et al*, 1988), ICP-34.5 (Martuza *et al*, 1991; Kesari *et al,* 1995; McKie, 1996) or thymidine kinase (Martuza *et al*, 1991). A separate, but related method of replication targeting has recently been developed, in which E1B deficient replicate with approximately 100 times greater efficiency in tumor cell lines with a functionally inactive p53 protein compared to p53 positive cells (Bischoff *et al*, 1996). The basis for this phenomonen lies in the normal function of E1B in allowing viral replication through binding and inactivation of p53. Reports of p53 deficiency in up to 30% of GBM indicates the potential for this methodology (Levine *et al*, 1991).

In the past, a large number of tumor or tissue selective promoters have been isolated and used to target transgene activation (Table 2). Difficulty has arisen when some regulatory sequences, the JC virus promoter for example (Paulus *et al*, 1996), appear to have lost tissue specificity when placed in viral constructs. This effect is likely the result of transcriptional override by strong native promoter

Table 2 Promoter sequences used to target transcription to tumor cells

Promoter	*Targeted tumor type*	*Reference*
Alpha-fetoprotein (AFP)	Hepatoma	Arbuthnot *et al*, 1995
Carcinoembryonic antigen (CEA)	Pancrease, lung, breast, colon	DiMaio *et al*, 1994 Osaki *et al*, 1994
Glial fibrillary acidic protein (GFAP)	Glioma	Besnard *et al*, 1991
JC virus immediate early promoter	Glioma	Henson *et al*, 1994
Myelin basic protein (MBP)	Glioma	Shimizu *et al*, 1994
Mouse mammary tumor virus	Breast	Arteaga and Holt, 1996
MUC1	Breast	Chen *et al*, 1995
Osteocalcin	Osteosarcoma	Ko *et al*, 1996
Prostate-specific antigen	Prostate	Pang *et al*, 1996 Lee *et al*, 1996
Surfactant protein A	Non-small cell lung cancer	Smith *et al*, 1994
Tyrosinase	Melanoma	Vile *et al*, 1994
Tyrosinase-related protein	Melanoma	Vile and Holt, 1993

sequences found in many viruses. Attempts are being made to delete or truncate suspect sequences (Ferrari *et al*, 1995; Vile *et al*, 1995).

With respect to gliomas, JC virus, glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) glial selective promoter elements have been characterized and used to drive targeted transgene expression. The JC virus promoter, which produces the glial tropism of the JC virus type that causes progressive multifocal leukoencephalopathy, is based on a glial promoter conserved guanine-rich GA box that binds the Sp1 transcription factor (MH-1 strain) (Hensen, 1994) and a TATA box flanking region that binds the glial cell protein Tst-1 (Mad-1 strain) (Krebs *et al*, 1994). This promoter has produced 30-fold higher transcription in glial compared to non-glial tumor lines (Hensen, 1994). When placed in a retroviral (Paulus *et al*, 1996) and HSV-1 amplicon (Pechan *et al*, 1996) vectors, however, extinction of this selectivity has been observed. GFAP is an intermediate-filament protein expressed selectively in reactive astrocytes and is used as a pathologic marker of astrocytic malignancies. The GFAP promoter (gfa2) has been demonstrated to elicit astrocyte-specific marker gene expression in culture and *in vivo* (Besnard *et al*, 1991; Brenner *et al*, 1994). HSV thymidine kinase (HSV-TK) driven by the MBP promoter and delivered by a retroviral vector has produced complete remissions in murine glioma models in conjunction with ganciclovir (Shimizu, 1994). Enhancer elements have also been found within the first and second introns of the nestin, an embryonal filament protein, which direct transcription to mitotic muscle and neural precursor cells (Zimmerman *et al*, 1994). The serotonin 2 receptor (Ding *et al*, 1993) and myelin proteolipid protein (Miyao *et al*, 1993) promoters are also selectively activated in glial cells. Use of promoter elements from the glioma associated antigens mentioned above may also be possible. The promoter region from tenascin, for example, has been characterized (Gherzi *et al*, 1995).

The potential for using general neoplasia and cell cycle-related promoters also exists. Promoter sequences have been characterized for VEGF (Tischer *et al*, 1991; Levy *et al*, 1995) (expression is increased 20–50 times in GBM compared to low-grade gliomas (Weindel *et al*, 1994)), proliferating cell nuclear antigen (PCNA) (Matsuoka *et al*, 1993) (expression correlates with aggressiveness in GBM (Schiffer *et al*, 1997)), basic fibroblast growth factor (bFGF) (Myers *et al*, 1995) (expression correlates with malignancy in gliomas (Takahashi *et al*, 1992)), transforming growth factor alpha (TGFα) (Shin *et al*, 1995) and c-*myc* (Kumagai *et al*, 1995). The grp78 (glucose-related protein) is induced by hypoxia, which is common in tumor tissue, and the promoter has been isolated and used to target transgene expression in a fibrosarcoma model (Gazit *et al*, 1995).

Transcriptional targeting of tumor vasculature could have the same potential benefits discussed with regards to receptor targeting, namely vascular deprivation and secondary infection of the associated tumor. Multiple vascular or neovasculature related promoters have also been characterized and utilized in therapeutic models. Ozaki *et al*, used the von Willebrand factor promoter to drive HSV-TK expression in human umbilical vein endothelial cells (HUVEC) using a retroviral vector (Ozaki *et al*, 1996). The ELAM and ICAM promoters are radiation inducible, ELAM selectively so within endothelial cells (Hallahan *et al*, 1996). Since neovascular endothelial cells also proliferate rapidly, some of the aforementioned cell cycle related promoters could target transcription to tumor neovasculature as well as tumor.

Various means further exist for activating genes in a temporally or spatially circumscribed manner. The most publicized of these are the radiation or oxidation state-inducible promoters such as those of EGR-1 (early growth response-1) (Datta *et al*, 1992) and tPA (tissue-type plasminogen activator) (Boothman *et al*, 1994). In addition to the ELAM and ICAM regulatory sequences discussed previously,

the IEX-1 (Kondratyev *et al*, 1996), c-*jun* (Sherman *et al*, 1990), Nfkβ (Brach *et al*, 1991), IL-1 (Ishihara *et al*, 1993), EGFR (Peter *et al*, 1993) and TNFa (Hallahan *et al*, 1989) genes also appear to be induced by radiation. Radiation inducible promoters have particular potential, since radiation plays a significant role in the treatment of most CNS malignancies and modern techniques allow precise delivery of radiation to the chosen target volume. Furthermore, radiation may increase the transduction efficiency of many vectors (Stevens *et al*, 1996), and acts synergistically with a number of common transgene systems. The EGR promoter has been used to regulate TNFa, as well as HSV-TK which activates the prodrugs ganciclovir, acyclovir and BVdU. Both systems sensitize cells to radiation in addition to having intrinsic toxicity (Kim *et al*, 1994, 1995; eischelbaum *et al*, 1994). The TNFa system has improved tumor growth delays and produced cures in experimental glioma models in conjunction with radiation (Hallahan *et al*, 1995). Promoter systems have also been designed which are regulated by levels of systemically administered pharmaceuticals such as tetracycline ((positive (Gossen *et al*, 1995) and negative (Gossen and Bujard, 1992) regulation)), estradiol (Braselmann *et al*, 1993), estrogen analogue (Whelan *et al*, 1996), progesterone analogues (Wang *et al*, 1994) and corticosteroid (Lu and Federoff, 1995). These promoter cassettes have also been combined with tissue selective promoters to create systems that are dually regulated to activate transcription under control of the pharmaceutical only in the target tissue (Fishman *et al*, 1994).

Promoters elements can be used not only to target transgene activation, but potentially selective *in situ* propagation of viral vector. Recently the possibility of controlling viral replication through regulation of replication-essential genes with these promoters has been described. Pechan *et al*, designed a system designated 'piggy back' in which an HSV-1 amplicon and recombinant virus are interdependent on each other for replication, as the amplicon carries the essential intermediate-early gene IE3 which has been deleted from the virus (Pechan *et al*, 1996). The construct was created with the intent of controlling IE3 expression, thus replication, through a regulatable promoter. In this system, selectivity of promoter sequences was reduced, presumably because of promiscuous HSV-1 enhancer sequences retained in amplicon. The JC promoter was utilized in the initial publication, and we have since placed EGR-1 and IEX-1 into amplicons (Spear *et al*, 1997). This type of system also provides a possible solution to some of the primary difficulties with carrier cells. In this case controlled or targeted activation of viral reproduction or transgene expression would be extraordinarily useful in limiting host cell toxicity before sequestration in the tumor, thus maximizing tumor exposure and minimizing systemic exposure to vector. The basis for a similar system has been created for adenovirus, in which case the E1A gene is deleted from the viral genome and carried in a separate plasmid (Goldsmith *et al*, 1994). Lymphocyte activation dependent promoters could also be used, such that TILs or other carrier lymphocytes contacting target antigens in a stimulatory tumor microenvironment would simultaneously activate viral production. Candidate regulatory sequences include those of the IL-2, IL-2 receptor, TNFa and interferon gamma (IFNg) genes (Yamada *et al*, 1987; Vitolo *et al*, 1992; Camp *et al*, 1996; Wang *et al*, 1995; Ioannides *et al*, 1992).

Conclusion

Each of the advances discussed in this review has moved the field closer to the goal of a disseminated tumor-selective vector capable of significantly improving the overall therapeutic ratio towards high grade gliomas and other CNS malignancies. Selective delivery of vector to the tumor or sites at risk for involvement increases exposure of malignant cells and decreases exposure of normal cells. Receptor targeting methodologies increase transduction or transfection of tumor and tumor associated cells, frequently while decreasing gene delivery to non-target cells. Targeted carrier cells have significant potential for selective delivery of viral vectors to disseminated tumor sites, including those that may not be accessible by direct or hematogenous delivery of naked vector. Specific promoter sequences have demonstrated ability to preferentially activate toxic transgene transcription in tumor tissues as opposed to normal tissues, and they have further potential to do the same for viral replication. Many of these targeting strategies may be used simultaneously in the same vector in order to further maximize toxicity to the tumor while minimizing toxicity to normal tissues. Similarly, a realistic ultimate objective is not the use of a single gene therapy vector alone with curative intent, but the inclusion of one or several vectors in combined modality therapy with complementary conventional therapies to obtain cures that we now narrowly miss, lengthen expected survival or improve quality of life, as previous incremental advances in conventional therapies have done.

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