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Gene transfer and delivery in central nervous system disease

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Gene transfer offers the potential to explore basic physiological processes and to intervene in human disease. The central nervous system (CNS) presents a fertile field in which to develop novel therapeutic modalities to treat intractable and pervasive malignant tumors and neurodegenerative disease. The extension of gene therapy to the CNS, however, faces the delivery obstacles of a target population that is postmitotic and isolated behind a blood-brain barrier (BBB). Approaches to this problem have included grafting of genetically modified cells to deliver novel proteins or introducing genes by viral or synthetic vectors geared toward the CNS cell population. Direct inoculation and bulk flow, as well as osmotic and pharmacological disruption, have been used to circumvent the BBB's exclusionary role. Once the gene is delivered, myriad strategies have been used to affect a therapeutic result. Genes activating prodrugs are the most common antitumor approach. Other approaches focus on activating immune responses, targeting angiogenesis, and influencing apoptosis and tumor suppression. At this time, therapy directed at neurodegenerative diseases has centered on ex vivo gene therapy for supply of trophic factors to promote neuronal survival, axonal outgrowth, and target tissue function. Despite early promise, gene therapy for CNS disorders will require advancements in methods for delivery and long-term expression before becoming feasible for human disease.

Key Words * gene therapy * brain tumor * blood-brain barrier * neurodegenerative disorder

Gene transfer offers a powerful and unique approach to the study and treatment of human disease. Developing new therapeutic modalities for the central nervous system (CNS) is particularly attractive because many clinical problems such as malignant brain tumors, Alzheimer's disease, Parkinson's disease, stroke, and craniospinal trauma incapacitate a large population and account for an annual cost to society in the hundreds of billions of dollars. Generalization of gene therapy to the nervous system, however, faces significant delivery obstacles. Neurons are postmitotic, and the parenchyma is protected by a blood-brain barrier (BBB). Moreover, regenerative goals are complicated by the distinctive roles played by neurons based on phenotype and their position within a complex circuitry. Despite early promise and heightened expectations, clinical trials have been unable to demonstrate unequivocal therapeutic benefit.[25] This review focuses on the problem of gene delivery in the CNS and surveys progress made in gene therapy for CNS malignancies and neurodegenerative disease.

VECTORS SYSTEMS

General Considerations

The features of an ideal vector system are the ability to infect a broad range of host cells, efficient delivery of the therapeutic gene, and stable expression of the gene after infection. Such a vector should elicit little immune response, offer tissue specificity, and should be able to be concentrated to high-titer preparations. Toward this end, three general types of gene therapies have been developed for the CNS. The most common is mutant derivatives of native viruses. Examples are the retrovirus and adenovirus. A second type is liposomes, or artificial vectors, typically consisting of lipid-protein and DNA complexes that allow delivery into cells. The third type is novel mixed virus recombinants, which harbor features of one viral system coupled with features of another to achieve a unique vector for improved gene transfer and expression.

Retroviral Vectors

Retroviral vectors are the best-characterized viral vector and the most common vector for non-CNS use and ex vivo approaches. Examples include the Moloney murine leukemia virus, the human immunodeficiency virus, and the Rous sarcoma virus. Retroviruses integrate and express genes only in proliferating cells, thereby limiting their use for brain tumor cells in the CNS. They can also be used on explanted cells to supply key proteins such as trophic factors for neuronal protection. For tumor therapy, retroviruses provide the advantage of specificity to dividing cells. Retroviruses integrate into the genome and can result in stable gene expression over many cell divisions. Although most retroviral titers are too low for efficient use, a new generation of retroviral producer cells appears to provide high titers for in vivo application. Some disadvantages include the possibility of insertional mutation of endogenous cellular genes and rapid inactivation of the viral genome resulting in low transduction efficiency.[12]

Herpes Simplex Virus

Along with adenoviruses, herpes simplex virus (HSV) has been the principal viral vector for CNS transfer. Essential genes are deleted, rendering them replication defective except when propagated on complementing cell lines. The neurotropism, high transgene capacity (30 kb), and ease of preparing high titers are all attractive features. Unlike retroviruses, HSV does not require cell division or integration into the host genome for gene transfer, making it a suitable vector for postmitotic neurons. As with all vector systems, there are several drawbacks. Use of heterologous promoters in HSV has been difficult because non-HSV-derived promoters are only transiently or aberrantly expressed.[70] In addition, safety is a concern, as HSV expresses viral antigens that can induce inflammation. Herpes simplex virus vectors can be neurotoxic and establish latency in nondividing cells. Removal of the HSV intermediate early genes may aid in solving this problem. For tumor therapy, **HSV** mutants have been shown to produce long-term survival in animal models of brain tumors.[7,41,45]

HSV Amplicon System

The plasmid-based amplicon consists of a eukaryotic expression plasmid modified by the addition of a HSV replication and cleavage/packaging sequence. The construct is placed into virions by transfecting a packaging cell line and subsequent superinfection with a replication-defective helper virus. The packaging cell line provides an environment for the generation of viruses that contain the desired amplicon sequence. Amplicons can direct gene expression in neurons and glia. Within the amplicon format, cellular promoters appear to yield longer-term expression and confer greater tissue specificity to transgene expression.[8] Titers of amplicon stock are still generally lower than with recombinant vectors

(10^6 - 10^7 particles/ml). Furthermore, as with other vector systems, the helper virus may elicit an immune response.

Adenoviruses

Adenoviruses can transfer genes to both neurons and glial cells.[37,65] The advantages of this vector system include high transfection efficacy and the ability to be concentrated to high-titer preparations. Further advantages include the capacity to accommodate large genes while avoiding the disadvantages of lytic infection and acute neuronal toxicity. Unlike retroviruses, insertional mutagenesis is uncommon. The primary disadvantage of adenoviruses is that cell infection occurs nonselectively. Recombinational events may produce hypervirulent strains. Adenoviruses also elicit strong immune responses. The virus-induced cytotoxic T-lymphocyte immune response not only results in transient transgene expression and tissue damage but precludes repeated administration. Future deletion of immunodominant epitopes may limit immune response and extend transgene expression.

Adenoassociated Viruses

Adenoassociated viruses are relatively new members among vector systems. Their primary advantage is safety. The parental wild-type virus is a defective parvovirus that requires helper functions from other viruses such as adenovirus to reproduce progeny, thereby limiting the potential for unwanted vector spread. Adenoassociated viruses are nonpathogenic and not associated with any known diseases. The lack of undesired viral gene expression mitigates immune rejection, a significant problem seen with other vector systems.[72] Particles are small and can infect tissues that larger viruses have difficulty penetrating. Adenoassociated viruses are resistant to many physical and chemical factors such as detergents, a wide range of pH, repeated freeze-and-thaw cycles, lyophilizing, and heat denaturation up to 60°C. As with adenoviruses and HSV, adenoassociated viruses can transfect nondividing cells with a broad host range. Transduction has been reported for up to 18 months.[33,43] Conditions for using an adenoassociated virus in the CNS still require optimization. Although producing high titers is possible, this task can be technically difficult. Adenoassociated viruses do not exhibit natural neuronal tropism, have a low transgene capacity (4.7 kb), and are frequently contaminated with adenoviruses. Production and assay systems are underdeveloped in comparison to retroviruses and adenoviruses.

Liposomes

Direct gene transfer by injection into tissue or by airway or systemic delivery can be performed using liposomes containing DNA. Encapsulation of plasmid DNA into liposomes protects the DNA from degradation and minimizes toxicity and immunogenicity while providing a mechanism for DNA transfer to target cells by lipid fusion. Liposomes offer ease of manipulation, safety, and stability on storage. Little work has been done in the CNS, with most efforts centered on cystic fibrosis and muscular dysfunctions. Early work has offered promise, demonstrating successful gene transfer into the mouse brain.[50] Nevertheless, liposomal gene transfer is still considerably inefficient compared with viral methods. Incorporation of DNA and gene expression occur only transiently; DNA condensation is required and transfer across the BBB remains problematic. Conventional liposomes are also rapidly taken up by the immune phagocytic cells.

VECTOR DELIVERY STRATEGIES

General Considerations

Whereas the delivery of genes at the molecular level commands much attention, the problem of bringing the vector to the target tissue is an often overlooked hurdle. The BBB is both a functional and an anatomical construct that allows little transcellular or pericellular transport. The barrier limits particles based on electrical charge, lipid solubility, and molecular weight. The problem of traversing the BBB has long plagued those applying pharmacological approaches to tumors, as many have observed the ineffectiveness of chemotherapy to affect cerebral metastases even when systemic sites have been eradicated.[60]

The BBB is not the only obstacle. High interstitial fluid pressure within the tumor may reduce entry of therapeutic agents.[32,38] Heterogeneity of microhemodynamics, pH, and microvascular architecture within the tumor can further complicate delivery.[64] Rapid complement inactivation in the peripheral circulation may also preclude transvascular delivery of viral vectors. Finally, the CNS does not tolerate extended flow interruption to allow a vector preparation to dwell within the target tissue. Without further advances, systemic administration of viral vectors appears unlikely to succeed in the CNS. Several alternatives have been studied.

Direct Inoculation

Direct parenchymal injection avoids the BBB and complement inactivation. This approach confers the advantages of limited systemic toxicity, use of small amounts of virus, and localized vector concentrations. Direct injection infects surrounding cells with a volume of transduced cells dependent on the dose and concentration of virus. Following direct inoculation of adenovirus or HSV, transduction is mostly limited to a 2-mm radius from the inoculation site.[6,14] Although considerable in the rat brain, this volume would produce little impact within the large volumes of human brain tumors. Given this limitation, direct inoculation appears unsuitable for treatment of disseminated brain tumors or the critical leading edge of even modestly sized tumors. By spreading through secondary and tertiary infection, HSV may solve this problem.

Convection-Enhanced Delivery

Convection-enhanced delivery improves the volume of distribution by applying a pressure gradient during infusion.[5] This method has augmented delivery of high- and low-molecular-weight tracers. Studies of microcrystalline iron oxide nanoparticles in the rat brain demonstrated that the dose of the agent is the primary determinant of distribution.[36] A fivefold increase in the amount of the agent that was delivered was associated with a three- to fivefold increase in distribution. Factors such as infusion time and infusate volume were less important. Convection-enhanced delivery has been successfully applied to the delivery of adenovirus and HSV.[47] Adenovirus spreads to an average volume of 40 to 60 mm³, whereas replication-conditional HSV spreads to a volume of 150 to 200 mm³.

Inoculation into Cerebrospinal Fluid

Direct injection of adenovirus into the cisterna magna results in gene transfer to the leptomeningeal cells overlying the major arteries, adventitial cells of large vessels, and occasionally to smooth-muscle cells of small vessels.[51] Gene transfer to cerebral vessels may be useful in studying vascular biology and allowing delivery of a transgene to aid in treating cerebral vasospasm. Intrathecal administration of an HSV vector transfects tumor cells that have undergone cerebrospinal fluid dissemination.[35] Introduction of a vector into ventricles may transfer genes to the ependymal cells and the choroid plexus. This method may provide a source of secreted products that enter the cerebrospinal fluid and penetrate

the brain.

Producer Cells

To increase the virus titer and exposure time at the tumor site, retrovirus producer cell lines have been grafted into cerebral tumors.[13,59] This approach has yielded effective transfection and tumor regression in animal models.[13,21] In human trials, early results indicate that tumors with virally infected cells were detected only 20 to 30 cell diameters from the transplant. This limited diffusion can be addressed by injecting cells into multiple sites to ensure an even distribution of virus throughout the tumor.[53] Clinically, this has been problematic because multiple passes through the brain have been required, thus increasing the complication rate.

Transvascular Delivery

Osmotic Manipulation. Osmotic disruption of the BBB may be an effective means to deliver recombinant viruses globally throughout the CNS. Osmotic disruption of the BBB separates tight junctions by shrinking endothelial cells. Mannitol is the most common hypertonic solution used for this purpose. Permeability is increased by 5 to 15 minutes postinfusion and normalizes within 2 hours.[54] In the rat, delivery of a viral vector across the BBB can be increased fourfold by using this approach.[49] Transgene expression has been demonstrated throughout the basal ganglia and cerebral cortex following osmotic disruption and intracarotid injection of HSV or adenovirus.[14,16] It appears that astrocytes may be targeted because their foot processes are responsible for the cuffing of vessels and for the integration of the BBB. In terms of safety, BBB disruption has been attempted in 300 patients and has not produced significant morbidity.

Bradykinin Analogs. Leukotrienes and vasoactive peptides can temporarily open the BBB for tumor therapy. Particularly attention has been directed at RMP-7, a B₂-receptor antagonist that has been shown to be 100-fold more potent than bradykinin in mice and is resistant to enzymatic degradation.[15] Intracarotid infusion of RMP-7 can increase uptake of low-molecular-weight agents such as methotrexate or carboplatin to brain tumors by nearly threefold and can increase dextran delivery by more than 10-fold.[31,42] The permeability of the BBB is greatest during bradykinin infusion (12-fold more than control) and returns to normal in approximately 20 minutes.[31] The BBB disruption is mostly limited to the tumor and neither bradykinin or RMP-7 significantly affects permeability around the tumor. In the RG-2 glioma model, rats treated with RMP-7 and carboplatin had significant increases in survival as compared with rats treated with carboplatin alone.[42] Bradykinin has also been used to enhance delivery of microcrystalline iron oxide nanoparticles and HSV particles in the 9L gliosarcoma model.[52] Delivery to the tumor is enhanced without infecting normal brain. Despite these successes, pharmacological manipulation of the BBB remains inconsistent. Although in the RG-2 glioma model, Inamura, et al.,[31] were able to demonstrate that bradykinin induced a 12-fold increase in uptake, they were unable to demonstrate significant changes in uptake in the 9L and C6 models. Others have found improved delivery in the 9L model (E. A. Chiocca, unpublished observations).

GENE THERAPY FOR BRAIN TUMORS

Genes used for the treatment of brain tumors can be divided into four groups: genes that 1) activate prodrugs; 2) activate immune responses; 3) modulate angiogenesis; and 4) are involved in apoptosis and tumor suppression.

Prodrug Activation

The prototype gene of this class is the **thymidine kinase (TK)** gene from the HSV type 1. The gene converts acyclovir and ganciclovir into competitive inhibitors of endogenous nucleotides for incorporation into the DNA chains of proliferating cells, leading to their death. The transfer of the **TK** gene confers lethal sensitivity to ganciclovir and has been successfully applied to animal models of brain tumors in vitro and in vivo.[3,10,13,62] In the "bystander" effect, death occurs in cells not transfected by virus but merely adjacent to successfully transfected cells, possibly by gap-junction-mediated transfer of ganciclovir metabolites in vitro. In vivo, the mechanism of the bystander effect may involve transfer of apoptotic vesicles or local inflammatory responses against virally infected tumor cells. A short list of other genes in this category includes those that activate chemotherapeutic agents. Examples include the **Escherichia coli guanine phosphoribosyltransferase** gene to activate 6-thioxanthine and 6-thioguanine;[46,63] the **E. coli cytosine deaminase** gene that activates 5-fluorocytosine;[48] the rat cytochrome **P450 2B1** gene that activates cyclophosphamide;[67,69] the **E. coli nitroreductase** gene that activates CB1954;[1] and the **deoxycytidine kinase** gene that activates cytosine arabinoside.[40]

Activating Immune Responses

Genes can be introduced that enhance antitumor immunity by their products. These include the **interleukin-4** gene and **antisense insulin growth factor 1** which have triggered rejection of glioma cells in rats.[66,68,73] Introduction of the gene for granulocyte-macrophage colony-stimulating factor may produce rejection of tumor cells by facilitating antigen presentation.[17] **Antisense transforming growth factor- β** has also been applied to the treatment of 9L gliosarcoma in Fischer 344 rats.[22]

Angiogenesis Modulation

Tumor neovascularization provides a target for gene therapy. Introduction of antisense vascular endothelial growth factor (VEGF) cDNA markedly inhibits the growth capacity of C6 glioma cells and reduces blood vessel formation.[57] Tumor involution has been achieved through introduction of a dominant-negative flk-1 (VEGF-R2) receptor into C6 glioma cells by means of a retroviral vector.[44] Antiangiogenesis may confer specificity because targeted molecules such as VEGF are limited to tumor blood vessels.[58] However, in vivo efficacy remains to be seen. To date, experiments have been performed on stably transfected cell lines and in vivo methods of gene transfer were not used. Moreover, successful interruption of one angiogenic pathway may select for tumor cells that depend on another avenue.

Apoptosis and Tumor Suppression

The understanding of tumor suppression genes such as **p53** and oncogenes such as **ras** identify them as potential targets for gene therapy. For example, an anti-**ras** approach using an adenoviral vector has demonstrated inhibition of proliferation and viability in bladder carcinoma cells.[23] Mutant **p53** genes, on the other hand, allow cells to proceed through the cell cycle and propagate errors in their DNA.[34] These errors may confer growth advantage and subsequent tumorigenesis. Adenovirus-mediated replacement of the mutant **p53** suppressor gene can elicit programmed cell death and regression of primary squamous cell tumors.[39] Sensitivity to **p53** expression has also been demonstrated in small cell lung carcinoma, prostate carcinoma,[71] and melanoma.[11] With respect to CNS disease, replacement of **p53** has been achieved through an HSV amplicon to a medulloblastoma line that bears a mutant **p53** gene.[56] An adenoviral vector has also been used to deliver wild-type **p53** to six

glioblastoma lines, resulting in inhibition of proliferation or apoptotic death.[27]

Clinical Studies

In the main clinical trial for gene therapy in brain tumors, the **HSV-tk** gene is delivered by retrovirus producer cells.[4] In the Phase I trial, patients received stereotactic inoculation of **TK**-retrovirus producer cells followed by a course of ganciclovir. Some patients exhibited partial regression in the early posttreatment period. Tumor biopsy samples revealed that **TK** was expressed in less than 1% of cells. "Bystander" mechanisms may explain any observed partial tumor regression. In the subsequent multicenter Phase II trial maximum resection of the tumor was achieved, followed by producer cell inoculation into the cavity wall, and reinjection by means of an Ommaya reservoir of producer cells every 40 to 45 days. Ganciclovir was administered after each producer-cell injection. Thus far, the average survival time for 18 patients was 25 weeks.[4] These studies formed the basis of the current prospective, randomized trial comparing standard treatment of a newly diagnosed glioblastoma with surgical tumor excision followed by injection of retrovirus producer cells and subsequent ganciclovir.

In addition to the retrovirus producer cells, a replication-defective adenovirus bearing the **TK** gene was used in a recent Phase I trial.[19] In this study, the tumor was inoculated with the **TK**-injected adenovirus followed by ganciclovir. Seven days after inoculation, the tumor was resected and the tumor cavity was injected with the adenovirus bearing the **TK** gene.

GENE THERAPY FOR NEURODEGENERATIVE DISEASES

The growing understanding that glial inhibition of axonal growth and deficits of trophic factors limit neuroregeneration has opened the door for reparative strategies.[24] The identification and cloning of molecules that influence neurodevelopment permit the use of gene transfer techniques in this arena. Thus far, the primary role for gene therapy has been ex vivo modification of cells to provide local trophic factors. In animal models of neurodegeneration neurotrophins such as nerve growth factor, brain-derived neurotrophic factor, fibroblast growth factor,[26] ciliary neurotrophic factor,[20] and neurotrophin-3[2] have been delivered using genetically modified fibroblasts to rescue neurons successfully.[55,61] Genetically modified cell lines have also been used to restore neurotransmitter supply. For example, cells modified to express tryptophan hydroxylase (TH) have shown promise in reducing symptoms of parkinsonism following implantation into animal models.[28,29,70] Likewise, fibroblasts engineered to produce acetylcholine promote neuronal survival in an animal model of Alzheimer's disease.

In situ approaches to gene transfer for neurodegenerative diseases have been less common. An HSV vector bearing the **TH** gene has produced behavioral improvements in an animal model of parkinsonism.[18] Similar results have been achieved with adenoviral and adenoassociated viral vectors carrying the **TH** gene.[30] In addition, an adenovirus expressing nerve growth factor has been shown to increase the somal area of acetylcholinesterase-positive cells in an animal model of senescence.[9]

CONCLUSIONS

Gene transfer techniques offer promise in treating a wide range of CNS diseases for which there is no effective treatment. By elucidating the mechanisms of CNS disease, gene therapy may also help to identify treatment strategies that may not require gene transfer. Nonetheless, the strong appeal of gene therapy in CNS diseases may obscure the many practical obstacles that remain to be worked out. Although identifying appropriate transgenes continues, methods for gene delivery in vivo still lack the degree of effectiveness needed to treat most human diseases. Current vector systems are hampered by

immunoreactivity and poor integration into the genome. Coupled with subsequent promoter silencing by DNA methylation, chromatin formation or genome sequestration, transgene expression is short-lived in most cases. Moreover, in vivo transfection can, at best, be achieved in only a minority of target cells. Even ex vivo approaches face rapid decline in transgene expression and limited graft survival, which preclude long-term effectiveness. The difficulty of transporting the vector across the BBB to the target tissue is an underappreciated problem. Most studies apply techniques that would provide too small a volume of distribution to be effective in the treatment of human diseases. Transvascular delivery awaits refinement in technique as well as production of high-titer vectors with little systemic toxicity. Once the difficulty of gene delivery and expression can be overcome, a number of transgenes appear to be attractive candidates for treating CNS malignancies and neurodegenerative disease.

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