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Gene Therapy Approaches to Parkinson's Disease

Andrew Freese

Department of Neurosurgery, Thomas Jefferson University, Philadelphia, Pa., USA

Over the past decade a number of improvements in gene therapy approaches to neurological diseases have made it possible that clinical trials for genetic intervention in patients with Parkinson's disease and other movement disorders will occur within the next several years, although significant obstacles persist that may restrain immediate application to patients. Approaches to neurological gene therapy can be divided into two broad categories: (i) ex vivo systems which are based on altering the genetic framework of cells or tissues and in turn these are grafted into the host brain and (ii) in vivo systems which are predicated on directly introducing therapeutic genetic information into desired locations within the brain, altering the host brain's physiology directly. Each of these systems has seen enormous advances, resulting in encouraging results in animal models of Parkinson's disease. These approaches will likely supplant tissue transplantation techniques that do not have the same scientific sophistication and have proven to be suboptimal and impractical in most clinical trials.

Ex vivo Approaches to Parkinson's Disease

Many studies have focused on the development of cell lines that express neuroprotective or neurorestorative genes, and these cell lines are then grafted directly into the brain, usually adjacent to or within the corpus striatum. The primary goal of these studies has been to introduce cells that function as a genetically engineered transplant that can secrete desirable substances that would ameliorate the symptoms associated with Parkinson's disease.

Most published studies have focused on introducing genes encoding the enzymes responsible for dopamine biosynthesis, including tyrosine hydroxylase

(TH) and aromatic amino acid decarboxylase (AADC or dopa decarboxylase), which respectively convert tyrosine into *L*-dopa, and in turn *L*-dopa into dopamine. More recently, studies have examined introducing cells transfected with alternative genes, including the gene encoding the enzyme (GTP cyclohydrolase) responsible for production of the cofactor for TH (tetrahydrobiopterin) and the gene encoding vesicular monoamine transporter, responsible for reuptake and storage of dopamine in the presynaptic terminal, augmenting its storage and neurotransmission. The hypothesis is that by introducing cells that secrete and increase levels of dopamine in the extracellular milieu, dopaminergic tone increases, and since dopamine appears to function more as a paracrine, slow-acting transmitter, an increase in dopamine tone is likely to have a beneficial effect on behavior. More recent studies have focused on genes that offer neuroprotective or even neuroregenerative functions, such as certain classes of growth factors, including glial cell line-derived neurotrophic factor.

Background of ex vivo Approaches

A number of groups, led by Gage et al. [45], first initiated studies in the mid-1980s focused on developing techniques to transfer genes into fibroblast cell lines. Early studies resulted in significant variability of transgene expression, which was largely due to the specific technique used for gene transfer, but the choice of promoter and other cellular factors also played a role in controlling gene expression. An early study by Palmer et al. [44], found that fibroblasts that had been transfected using retrovirus vectors had strong transgene expression, but this expression downregulated by over 1,000-fold within 1 month of transplantation into a host animal. Immunological rejection was not the cause of this downregulation, but rather instability of the regulatory elements was determined to be the cause [44]. In another report, Scharfmann et al. [48] found that long term gene expression could be achieved with an endogenous promoter, such as the housekeeping dihydrofolate reductase promoter, in place of viral promoters, and current studies have expanded the armamentarium of endogenous promoters used [24, 25], largely focusing on endogenous cellular promoters.

Immortalized Cell Lines

Immortalized cell cultures provided an initial focus for the development of an appropriate cell type for grafting into the recipient brain. At first, immortalized fibroblast cell lines [55] were used because they were easy to grow and could readily be genetically manipulated in tissue culture. A rat fibroblast cell line (208F) was transfected with the TH cDNA, and in vitro in the presence of the TH cofactor, tetrahydrobiopterin, *L*-dopa was synthesized and secreted extracellularly. In turn, these modified cells were grafted

into the brains of rats previously treated with 6-hydroxydopamine (6-OHDA), a neurotoxin that is used to create the rat rotational model of Parkinson's disease. Two weeks after grafting, a small reduction in the abnormal rotational behavior of the rats was observed, but immunohistochemical staining failed to show any TH staining, and a significant immunological response was identified, presumably due to a mismatch between host and graft. Thus, some of the behavioral effect may have been due to the induction of a lesion. Based on these and other studies, it has become quite clear that the brain is not 'immune-privileged' and therefore the immune system is an important constraint in any tissue grafting paradigm. Therefore, another study used immortalized, syngeneic Fischer fibroblast cell lines which were transfected with the TH gene and grafted into 6-OHDA-treated rats. Unfortunately these cells were found to form rapidly expanding tumors that ultimately killed the test animals. From this it became clear that cells that divide and form tumors cannot be the basis for ex vivo genetic manipulation.

Primary Culture Approaches

Over the past decade, numerous additional studies have developed improved approaches towards defining the appropriate tissue source for grafting. Initially much attention focused on immortalized cell lines, but more recent attention has focused on primary cell cultures since they are likely to be dormant within the postmitotic host brain and not form expanding tumor masses. In addition, much attention is now focused on the use of 'stem' cells or neural precursor cells derived from the subependymal zone, and other immortalized human stem cell-derived platforms for gene transfer and implantation.

Kawaja and Gage [32] studied striatal implantation of cultured primary skin fibroblasts in Fischer 344 rats. These fibroblasts lasted at least several months when transplanted into the host brain. In culture, these cells grow robustly, but once they form a confluent monolayer, growth arrest occurs. Similarly, in situ in the brain, these cells form a capillary network, a rudimentary blood-brain barrier, and a dormant tissue mass which can secrete substances into the adjacent host tissue. When such fibroblasts were transfected with the TH gene and grafted into the 6-OHDA rat rotational model, the grafts expressed the transgene and survived for more than 10 months. In tissue culture and in situ in the brain, *L*-dopa secretion was observed, and the 6-OHDA rats demonstrated significant behavioral recovery. Other related studies have expanded the potential role of such isogeneic cells that do not form expanding tumors to express the TH gene and amplify *L*-dopa, and presumably dopamine, formation in situ.

An alternative gene target has been the focus of more recent studies [30, 52]. The gene encoding the enzyme responsible for biosynthesis of the

TH cofactor, tetrahydrobiopterin, GTP cyclohydrolase (GTPCH1), in combination with the TH gene were transfected into primary fibroblasts, and these cells were grafted into the 6-OHDA rat model, with evidence of amplified *L*-dopa and dopamine biosynthesis and behavioral recovery. In addition, by combining the gene encoding the vesicular monoamine transporter [2] and aromatic amino acid decarboxylase in a construct introduced into primary skin fibroblasts, a significant increase in dopamine storage and behavioral recovery in parkinsonian rats were observed.

Numerous alternative cell types have been applied as well towards developing ex vivo approaches to genetic intervention. Included among these are autologous muscle grafts [27–29], Schwann cells [42], certain tumor cells lines [40], pig xenograft tissue, and biohybrid implants [38], as well as human stem cells. Similarly, alternative genetic targets have been identified, including glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). Studies using cell lines that secrete these substances have suggested a neuroprotective and potentially neuroregenerative effect on the nigrostriatal system [16, 23].

Disadvantages of the ex vivo Approaches

There are a number of advantages of ex vivo approaches to genetic intervention in neurological disorders, such as Parkinson's disease. These include a clear identification of the genetic elements that drive gene expression, unlike some of the in vivo approaches based on viruses that have less well characterized genetic elements. The concept of using grafted cells that act as 'factories' that secrete desirable products appears to be particularly appealing. Current technology permits long-term survival of the grafted cell lines, without producing tumors that kill the host.

There are, nonetheless, significant problems with ex vivo approaches. A tissue mass is grafted into the host brain which disrupts normal host circuitry and which does not integrate into the host circuitry. Cells that can divide or migrate may form masses or disrupt appropriate anatomy and/or physiology of the host brain. Endogenous control elements that regulate homeostasis are therefore bypassed, and regulation of the secreted substance becomes a problem. Because of the focal location of the tissue graft, diffusion of the secreted substance is essential, and for most substances, this diffusion is suboptimal and may not reach the desired target tissue. The long-term outcome from application of the most recent cellular grafts, such as the porcine cells and human stem cells, in humans for the former and in animal models for the latter, are still uncertain. Furthermore, in many studies using cellular-based grafts, scale-up from the rat 6-OHDA model to primates and eventually to humans has proven problematic.

In vivo Systems

In part because of the disadvantages of ex vivo approaches, particularly early on when grafted tissues formed expanding tumors, and because of the advent of new technology that permits direct gene transfer into target tissues, an alternative approach from ex vivo gene therapy has emerged, and has progressed in parallel, often intersecting the ex vivo systems whenever transgene delivery is desired. Because of the inherent elegance of in vivo approaches, and the increased sophistication of gene transfer technology, it is possible that in vivo approaches will prevail in clinical application of gene therapy for Parkinson's disease and other movement disorders, although such predictions are often proven incorrect as technology progresses

Gene Transfer Techniques: Background

A myriad of techniques has been used to transfer genes into mammalian cells, including use of liposomes, plasmids, viruses, and direct DNA transfer [10, 15]. Although the technology is advancing rapidly for nonviral systems, in general gene transfer vectors based on DNA viruses appear to hold the greatest promise for direct genetic intervention in Parkinson's disease. These viruses can transfect the postmitotic CNS, typically have large enough genomes to accommodate most therapeutic genes, and have been sufficiently attenuated to prevent a toxic infection in the host brain. At this point, three virus vector systems have been successfully applied to animal models of Parkinson's disease, resulting in a number of publications, although studies applying novel lentivirus vectors and nonviral liposome systems are ongoing. The three viruses that have been used are (i) herpes simplex virus-1 (HSV-1); (ii) adenovirus, and (iii) adeno-associated virus (AAV). Each of these has advantages and disadvantages, although it appears that AAV vectors are likely to reach clinical application first.

HSV-1 Vectors

There are a number of features of HSV-1 that make it an attractive vector for gene delivery into the brain. These include its wide host range in numerous neuronal and glial cell types, its regulated expression of genes, its ability to infect postmitotic cells, its large genome that can accommodate most therapeutic genes, its ability to remain quiescent in a latent state, and its absence of toxicity in the latent state [3, 4, 10–15, 17–20, 39, 43, 49].

There are two categories of HSV-1 vectors that have been developed for CNS gene transfer [10, 15]: (i) defective viral vectors and (ii) recombinant vectors.

Defective viral vectors are based on defective interfering HSV-1 particles, which occur when HSV-1 is grown at high multiplicities of infection (moi, or

the ratio of the number of viral particles to the number of cells). Defective interfering particles possess only a portion of the entire 150 kb HSV-1 genome, but this portion is repeated until approximately 150 kb of DNA is generated and this is then packaged into an HSV-1 virion. Defective interfering particles need help from wild-type HSV-1 since they cannot replicate and package their own DNA, but they do contain an HSV-1 origin of replication and the packaging signals needed for packaging the DNA into a virion [49, 53, 54]. Spaete and Frenkel [49] developed a prototype plasmid, termed an amplicon, which eliminated most of the native HSV-1 DNA and replaced it with a foreign transgene, but allowed packaging of the DNA into virus particles. This amplicon packaged multiple copies of the gene of interest in one particle, allowing these multiple copies of the transgene to gain access to the desired target cell. The resulting packaged viral vector was termed a defective viral vector, which contains a gene of interest packaged into an HSV-1 coat which cannot replicate on its own and which elicits minimal immune responses since it contains no or only a few HSV-1 proteins. A disadvantage is that a helper virus is required to provide the missing proteins required for gene therapy applications, and this helper virus can induce an immune response or prove to be toxic or even cause a lytic herpes infection.

The other type of HSV-1 vector is the recombinant vector, or replicon, in which one or more essential genes are eliminated from the HSV-1 genome. This, in turn, reduces the ability of the virus to replicate and cause toxicity in host cells or tissues. A new transgene, or gene of interest, can replace the gene(s) which has been eliminated, and now the transgene is expressed in host cells. Although most of these replicons have been developed with a solitary gene deletion, such that most of the other approximately 70 HSV-1 proteins are still made, newer systems are being developed which have numerous deletions, which further debilitate the virus' ability to damage tissue and reduce its immunogenicity. Unfortunately, because the majority of people have latent HSV-1 infections residing within their trigeminal ganglia, the potential for recombination between the vector and the endogenous wild-type virus is problematic, since it could result in a fatal encephalitis.

Early Studies with HSV-1 Vectors

A number of studies in the late 1980s used defective HSV-1 vectors [11, 12, 14, 17, 18, 20, 33] to demonstrate that they could transfect dividing cells and postmitotic neurons in tissue culture, resulting in transgene expression. Once the principle was established, a novel HSV-1 amplicon vector system was devised by Geller et al. [8, 10], which contained the cDNA expressing human TH (type II). This vector directed expression of human TH RNA and protein in neonatal rat striatal cells in culture [8, 10]. After

several days of transfection, cellular RNA was extracted and the presence of human TH mRNA was determined using reverse transcriptase polymerase chain reaction. This vector was also shown to direct expression of human TH protein in these cultures, as assayed by immunohistochemical techniques; most of the transfected cells were neurons. Both *L*-dopa and dopamine release were shown to increase in cells transfected with the experimental vector, compared to controls, and the release of these catecholamines was stimulated by physiologically depolarizing conditions. It is of note that the amount of TH enzyme produced was similar to that endogenously found in native catecholaminergic neurons [8, 10], and *L*-dopa production was comparable to that found in genetically engineered cells (for ex vivo gene therapy studies) transfected with TH.

In vivo Studies

After establishing that an HSV-1 vector could direct TH expression and *L*-dopa production in cultured neostriatal neurons, attention was focused on applying this vector to the 6-OHDA rat rotational model [8]. In those rats which received the TH vector directly introduced stereotactically into the striatum, compared to controls, there was an approximately 65% reduction in aberrant rotational behavior, sustained for 1 year. Microdialysis studies directly measuring levels of dopamine in situ in these rats revealed significant increases in extracellular dopamine levels, which responded to physiological depolarizing conditions. Furthermore, immunocytochemical evaluation for TH expression in sacrificed rats showed numerous cells, mostly neurons, expressing human TH in those animals transfected with the TH vector, and not seen in controls. These results indicated that the experimental vector directed human TH gene transfer into the rat brain, which resulted in transcription, translation, and expression of a functional TH protein that increased *L*-dopa and dopamine production. This resulted in a sustained and significant behavioral improvement in 6-OHDA-treated rats. Unfortunately, in this study, some rats succumbed to HSV-1 encephalitis, presumably mediated by reversion of the vector system to wild-type virus. These results were the first demonstration of efficacy of an in vivo gene therapy approach to Parkinson's Disease, but also highlighted the significant problems with HSV-1 vectors, and viral gene transfer in general.

Newer HSV-1 Vectors

More recently approaches have been developed to reduce the cytopathic effects of HSV-1 vectors. Cell type-specific factors, such as Oct-2 [26], have been used to interfere with HSV lytic infections. Novel recombinants with multiple deletions have been developed that appear to reduce neurovirulence

[22]. Newer, helper-free amplicon vector stocks have been developed which appear to reduce cytotoxicity as well [21]. However, there remains significant reluctance to use HSV-based vectors for restorative molecular gene therapy because of the concerns of immunogenicity and cytoxicity, largely related to the risk of recombination and production of endogenous, immunogenic viral proteins.

Adenovirus Vectors

Because of the established cyototoxicity of HSV-1 vectors, other virus vector systems have been explored for potential application to Parkinson's disease. In 1993, a number of publications suggested the potential of adenovirus vectors to direct gene transfer into the mammalian brain [1, 2, 5, 34]. In most of the studies, it appeared that glial cells were predominantly targeted, but neurons appeared to express the transgene as well. At low titers, minimal toxicity was seen, whereas at higher titers, significant evidence of neurotoxicity was observed. Although in the short term (up to 1 month), a large number of cells were transduced and showed transgene expression, at 2 months most of the gene expression had disappeared.

Based on these encouraging preliminary studies, a number of groups have used adenovirus vectors to direct TH transfer and expression in striatal cells, both in tissue culture and in the 6-OHDA rat rotational model. In one study, a significant reduction in rotational behavior was observed for just 2 weeks; in another study, longer term efficacy was demonstrated [24, 25]. In a recent study, Leone and co-workers [unpubl. results] have recently found that there is a direct linear correspondence between the number of cells which express TH following adenovirus vector-mediated gene transfer and behavioral recovery in rats.

Additional studies have focused on gene transfer of neuroprotective substances using adenovirus vectors. Human GDNF cDNA was introduced into substantia nigra cells, resulting in increased sprouting and survival of these cells. Further definition of the role of such neuroprotective strategies using adenovirus-mediated gene transfer is ongoing.

A significant obstacle in the application of adenovirus vectors to CNS gene therapy is their inherent immunogenicity. Although newer generation adenovirus vectors appear to have reduced induction of an immune response, in most studies this has proven to limit long-term expression and induce inflammatory responses in target tissues, including the brain. Human trials using adenovirus vectors for application to CNS malignancy has revealed that the immune response in the human brain is quite significant, and more effective strategies need to be developed to obviate this issue before application to human patients with Parkinson's disease may be considered.

Adeno-Associated Virus Vectors

Based on the limitations of HSV-1 and adenovirus vectors, a number of investigators have focused their attention on an alternative viral vector, based on AAV [13, 31]. AAV is a nonpathogenic DNA virus which is incapable of autonomous replication and spread, and which can, under certain circumstances, integrate into the host chromosome, increasing transgene stability [31, 41, 47].

Vectors based on AAV can be produced to be entirely free of helper viruses and do not express any endogenous viral proteins. This reduces the likelihood of toxicity or immunogenicity, and offers a significant advantage over other DNA viruses which retain the ability to produce endogenous viral proteins. In all published reports using AAV vectors, there is no evidence of cytopathic effects, in sharp contrast to studies using adenovirus and HSV-1. There are some disadvantages for AAV, however. These include observations that viral titers are lower than those obtained with adenovirus and HSV-1. The AAV genome can only accommodate 5 kb or smaller transgene sequences, in contrast to HSV-1 which has a much larger genome and can therefore incorporate larger transgene sequences [10, 15].

A number of investigators have performed studies over the past several years examining the potential application of AAV vectors for therapy in Parkinson's disease. During and co-workers [31] developed an AAV vector expressing human TH (form II) cDNA, which caused significant expression of TH in cultured striatal neurons. The majority of transfected cells were neurons, although glial cells also expressed human TH. Elevation of *L*-dopa synthesis was observed, leading to application of this vector to the 6-OHDA rat rotational model of Parkinson's disease. Injection of the AAV vector expressing TH resulted in a sustained and significant recovery of apomorphine-induced asymmetrical rotation for several months, whereas control vector or buffer injections had no effect.

A more recent study [9] used an AAV vector which not only expressed human TH, but also AADC cDNA. By expressing both of the enzymes responsible for dopamine biosynthesis, the hypothesis was that there would be even further amplification of dopamine production, since this strategy would not have to rely on endogenous decarboxylase activity. This vector was then applied to the MPTP primate model of Parkinson's disease. In the experimental animals which received the TH/AADC vector, a number of molecular biological techniques demonstrated the presence of human TH and AADC, which were not found in control animals. Although the number of primates was small, in some animals a significant and sustained behavioral recovery was noted, which in some cases was quite dramatic. Expansion of this study to a larger animal cohort is planned.

Another study [6, 7] evaluated an AAV vector expressing glial cell line-derived neuronotrophic factor (GDNF). GDNF has been shown to be a neurotrophic factor that promotes midbrain dopaminergic survival [35, 37, 51]. In embryonic mesencephalic rat cells in culture, the AAV vector expressing GDNF resulted in increased survival of dopaminergic neurons. Similarly, when applied to animal models, there was evidence of neuroprotection. Two animal models of nigral degeneration were used. In the first, rats had prelabeling of nigrostriatal neurons performed with fluorogold; subsequently bilateral 6-OHDA lesioning was induced. The AAV vector expressing GDNF was introduced unilaterally prior to introduction of 6-OHDA, and significant neuroprotection was observed on this side, with increased striatal dialysate levels of dopamine and rotational effects. Similarly, in two primates treated with MPTP, stereotactic introduction of AAV-expressing GDNF unilaterally resulted in increases in single-photon emission-computed tomography (SPECT)-based imaging of the dopamine transporter on the treated side, which was not seen on the control side.

Ideally, a combination of neuroprotective and neurorestorative genetic intervention would be available. Therefore, studies are currently focused on developing novel vectors that express both enzymes involved in dopamine biosynthesis, as well as proteins that are neuroprotective. Included among the target-restorative genes currently being evaluated are TH, AADC, GTP cyclohydrolase, and antisense to the dopamine transporter. Included among target neuroprotective/neuroregenerative genes being studied are GDNF, BDNF, Nurrl, and other members of different neurotrophin families.

Problems with Gene Therapy for Parkinson's Disease

Although significant advances have been made in the development of gene therapy approaches to Parkinson's disease, and other neurological diseases, there still remain significant obstacles that preclude immediate application to human patients of these approaches. Among these obstacles are (i) the need to define the most appropriate transgene and promoter; (ii) identification of which vector or cell line should be applied; (iii) optimization of the delivery system; (iv) definition of the target tissue, and (v) assurance of no toxicity or immune response. It is incumbent upon investigators to avoid the mistakes of the tissue transplant field, which rushed prematurely into clinical trials, at first leading to a debacle with autologous adrenal transplants that had no efficacy and evidence of toxicity, and more recently the significant evidence that fetal transplants also may cause significant morbidity, worsen parkinsonian symptoms, and have no meaningful long-term efficacy.

The ideal choice of transgene in gene therapy for Parkinson's disease has not yet been decided. Even if one favors a dopaminergic restorative approach, should one use TH, AADC, GTP cyclohydrolase, vesicular monoamine trans-

porter, or a combination thereof? No study has directly compared these transgenes in animal models, and clearly such a study must be done prior to moving into human clinical trials. The choice of promoter is also uncertain. A plethora of promoters has been used in a number of studies, and it does appear that endogenous cellular promoters are the optimal choice, but these even vary, in part dependent on the cell types that are transfected, the brain location that is chosen, and the amount and longevity of expression that is achieved. Ultimately, comparative analysis of different promoters in must be undertaken before moving into clinical trials.

The ideal choice of viral (or nonviral) vector or cell line used for gene transfer remains unclear, and is largely driven by the selected experience of individual research laboratory groups rather than an objective understanding of what the best system may be. Furthermore, as more biotechnology companies are focusing on the significant economic potential of genetic intervention in neurological diseases, they will tend to promote their own proprietary technology, rather than identifying the most appropriate system for human application. Ultimately, to avert a potential disaster, the scientific and medical community must develop its own consensus about which approach to first use in human patients, unfettered by economic considerations.

The delivery system used to introduce into the brain cell lines or genetic vectors is still an area that needs much investigation. Simple stereotactic delivery appears to be the most appropriate, but questions of volume of injection, rate of injection, number of injections, use of drugs to alter the extracellular fluid compartment and blood-brain barrier (such as mannitol), and other issues remain unsolved. Alternatively, endovascular approaches using newer generations of catheters that could target select brain regions may provide an elegant alternative to invasive surgery.

The optimal choice of target tissue is still uncertain. Is the corpus striatum the right choice? Should attention be focused on the substantia nigra, or even the subthalamic nucleus? Comparative studies have not yet been performed, and these must be done prior to rushing into human trials.

Finally, the issue of cytoxicity and immune responses, particularly to viral vectors, is paramount. Because of the sequestered nature of the target tissues, and the limited flexibility of the nigrostriatal system, particularly in a fragile Parkinson's disease patient, it must be clearly established that whatever system is used cannot lead to problems, even in the long term. Since there are attractive pharmacological and surgical options for patients with Parkinson's disease, before clinical trials are undertaken, it is an ethical responsibility of investigators to ensure the safety of any gene transfer approach to these patients. Long-term studies in animal models must therefore be done, and focusing on safety and efficacy in patients should be paramount.

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Dr. Andrew Freese, Department of Neurosurgery, Thomas Jefferson University, Suite 1400, 1015 Chestnut Street, Philadelphia, PA 19107 (USA)
Tel. +1 215 955 0951, Fax +1 215 955 0957