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RESTORATIVE GENE THERAPY APPROACHES TO PARKINSON'S DISEASE

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Numerous advances over the past decade have made it likely that clinical trials for genetic intervention in patients with Parkinson's disease and other movement disorders will occur within the next few years. Approaches to neurologic gene therapy include (1) ex vivo systems, based on altering the genetic framework of cells or tissues and then grafting these into the host brain, and (2) in vivo systems, predicated on directly introducing therapeutic genetic information into the brain. Each of these systems has seen enormous advances, resulting in encouraging results in animal models of Parkinson's disease. These approaches are likely to supplant tissue transplantation techniques that do not have the same scientific sophistication and have proven to be suboptimal in most clinical trials.

EX VIVO APPROACHES TO PARKINSON'S DISEASE

A variety of studies have focused on the development of cell lines that contain neuroprotective or neurorestorative genes, which are then grafted directly into the brain, usually adjacent to or within the corpus striatum. The primary goal of these studies was to introduce cells that function as a transplanted *factory* that can secrete desirable substances that ameliorate the symptoms associated with Parkinson's disease.

Most published studies have focused on introducing the genes

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encoding the biosynthetic enzymes responsible for dopamine biosynthesis, tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC or dopa decarboxylase), which convert tyrosine into L-dopa and L-dopa into dopamine. The hypothesis is that by introducing cells that secrete dopamine into the extracellular milieu, dopaminergic tone increases, and because 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.

Background of Ex Vivo Approaches

Ray and Gage[45] and other groups 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, in part, due to the specific technique used for gene transfer, but the 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 down-regulated by more than 1000-fold within 1 month of transplantation into a host animal. Immunologic rejection was not the cause of this down-regulation, 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]

Immortalized Cell Lines

Immortalized cell cultures were the initial focus of research scientists interested in developing an appropriate cell type for grafting into the recipient brain. Initially, 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 immunologic response was identified, presumably resulting from a mismatch between host and

graft. Based on these and other studies, it has become clear that the brain is certainly not *immune-privileged*, and the immune system is an important constraint in any tissue grafting paradigm. Because of this failure, another study used immortalized, syngeneic Fischer fibroblast cell lines, which were transfected with the TH gene and grafted into 6-OHDA-treated rats. 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 toward 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 because they are likely to be dormant within the postmitotic host brain and not form expanding tumor masses.

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 that 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, guanosine triphosphate (GTP) cyclohydrolase (GTPCH1), in combination with the TH gene was 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.

Numerous alternative cell types have been applied as well toward developing ex vivo approaches to genetic intervention. Included among these are autologous muscle grafts,[27–29] Schwann's cells,[42] certain tumor cell lines,[40] pig xenograft tissue, and biohybrid implants.[38] 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]

Problems with the Ex Vivo Approaches

There are a number of advantages of ex vivo approaches to genetic intervention in neurologic disorders, such as Parkinson's disease. Advantages include a clear identification of the genetic elements that drive gene expression, in contrast to 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.

Nonetheless, there are significant constraints 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. Endogenous control elements that regulate homeostasis are therefore bypassed, and regulation of the secreted substance becomes problematic. 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. Scale-up from the rat 6-OHDA model to primates and eventually to humans has proven problematic.

IN VIVO APPROACHES

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 techniques that allowed direct gene transfer into target tissues, an alternative approach from ex vivo gene therapy has emerged and has progressed in parallel. Because of the inherent elegance of in vivo approaches and the increased sophistication of gene transfer technology, in vivo approaches are likely to prevail in clinical application of gene therapy for Parkinson's disease and other movement disorders.

Background of Gene Transfer Techniques

A variety of techniques have been used to introduce genes into mammalian cells, including 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. Such viruses can transfect the postmitotic central

nervous system, usually have large enough genomes to accommodate most therapeutic genes, and have been 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, although application of novel lentivirus vectors and nonviral liposome systems are imminent. The three viruses that have been used are (1) herpes simplex virus-1 (HSV-1), (2) adenovirus, and (3) adeno-associated virus (AAV). Each of these has advantages and disadvantages, although it appears that AAV vectors are likely to reach clinical application first.

Herpes Simplex Virus-1

There are a number of features of HSV-1 that make it an attractive vector for gene delivery into the brain. Included among these are its wide host range in numerous neuronal and glial cell types, its ability to infect postmitotic cells, its regulated expression of genes, 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 central nervous system gene transfer[10, 15]: *defective viral vectors* and *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 (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 because 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 Frankel[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 that cannot replicate on its own and that elicits minimal immune responses because it contains no or only a few HSV-1 proteins. A disadvantage is that a helper virus is needed to provide the missing proteins required for gene therapy applications, and this helper virus can induce an immune response or prove to be toxic.

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 that 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 that have numerous deletions, which further debilitate the virus' ability to damage tissue and reduce its immunogenicity. 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, as it could result in a fatal encephalitis.

Early Studies with Herpes Simplex Virus-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 During et al[8] and others[10] that 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 with controls, and the release of these catecholamines was stimulated by physiologically depolarizing conditions. 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

Once it was established that an HSV-1 vector could direct TH expression and L-dopa production in cultured neostriatal neurons, attention was directed to the 6-OHDA rat rotational model.[8] In rats that received the TH vector directly introduced stereotactically into the striatum, compared with controls, there was an approximately 65% reduction in aberrant rotational behavior, which was sustained for 1 year. Microdialysis studies in these rats revealed significant increases in extracellular dopamine levels, which responded to physiologic depolarizing conditions. Postmortem immunocytochemical evaluation of TH showed numerous cells, mostly neurons, expressing human TH in the rats transfected with the TH vector, which was 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. In turn, this resulted in a sustained and significant behavioral improvement in 6-OHDA-treated rats. 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.

Newer Herpes Simplex Virus-1 Vectors

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 that appear to reduce cytotoxicity as well.[21]

Adenovirus Vectors

Because of the established cytotoxicity of HSV-1 vectors, other virus vector systems have been explored for potential application to Parkinson's disease. In 1993, a number of reports indicated 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] Leone et al (unpublished results) have found that there is a direct linear correspondence between the number of cells that express TH after adenovirus vector–mediated gene transfer and behavioral recovery.

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 central nervous system 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.

Adeno-associated Virus Vectors

As a result of 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 that is incapable of autonomous replication and spread and that can, under certain circumstances, integrate into the host chromosome, increasing transgene stability.[31, 41, 47]

AAV vectors can be generated to be entirely free of helper viruses and do not express any endogenous viral proteins. This characteristic 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 accommodate only 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 4 years examining the potential application of AAV vectors for therapy in Parkinson's disease. Kaplitt et al[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. Elevations of L-dopa synthesis were observed, prompting 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 asymmetric rotation for several months, whereas control vector or buffer injections had no effect.

One study[9] used an AAV vector that 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 because 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 that received the TH/AADC vector, a number of molecular biologic 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 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 before 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–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 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 and neuroregenerative genes being studied are GDNF, BDNF, Nurrl, and other members of different neurotrophin families.

SUMMARY

Perhaps one of the most exciting developments in brain research of the past decade is the advent of genetic intervention in human neurologic disease. Although there are a variety of gene transfer approaches, none of which has been perfected, gene therapy is now science *fact* and no longer science fiction. As technology progresses, some vectors will prove more effective for certain disease categories than others; it is too early to predict definitively which vector would be most effective for therapy in Parkinson's disease and other movement disorders. Nonetheless, it is likely that within the next year or two a gene therapy trial will be instituted in human patients with Parkinson's disease. The potential for an impact on the symptoms and progression of this disease is significant. Clinicians may be on the threshold of a new era of intervention for Parkinson's disease and other neurologic diseases, based on bypassing traditional but less selective drug–extracellular receptor interactions and instead focusing on genetic modulation of specific intracellular processes. The continuing development of small incremental changes of

new dopamine agonists and pharmacologic agents will likely pale in comparison to the specificity of intracellular genetic manipulation.

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