**In utero** gene transfer to the mouse nervous system

Ahad A. Rahim*, Andrew M.S. Wong†, Suzanne M.K. Buckley‡, Jerry K.Y. Chan§, Anna L. David*, Jonathan D. Cooper†, Charles Coutelle∥, Donald M. Peebles‡ and Simon N. Waddington*†

*Institute for Women’s Health, University College London, 86-96 Cheyne Mews, London WC1E 6HX, U.K., †Paediatric Storage Disease Laboratory, Institute of Psychiatry, Kings College London, 125 Coldharbour Lane, London SE5 9NU, U.K., ‡Department of Haematology, University College Medical School, Rowland Hill Street, London NW3 2PF, U.K., §Division of Maternal and Fetal Medicine, Experimental Fetal Medicine Group, Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, and ¶National Heart and Lung Institute, Imperial College London, South Kensington, London SW7 2AZ, U.K.

Abstract

The cellular and molecular environment present in the fetus and early newborn provides an excellent opportunity for effective gene transfer. Innate and pre-existing anti-vector immunity may be attenuated or absent and the adaptive immune system predisposed to tolerance towards xenoproteins. Stem cell and progenitor cell populations are abundant, active and accessible. In addition, for treatment of early lethal genetic diseases of the nervous system, the overarching advantage may be that early gene supplementation prevents the onset of irreversible pathological changes. Gene transfer to the fetal mouse nervous system was achieved, albeit inefficiently, as far back as the mid-1980s. Recently, improvements in vector design and production have culminated in near-complete correction of a mouse model of spinal muscular atrophy. In the present article, we review perinatal gene transfer from both a therapeutic and technological perspective.

Introduction

Early lethal genetic diseases of the nervous system are individually rare, yet collectively numerous and, in some populations, high prevalence values have been reported [1]. A subset of these comprises lysosomal storage diseases such as acute neuropathic (Type II) Gaucher disease, neuronal ceroid lipofuscinoses and Niemann–Pick disease type C. In many cases, prognosis is dismal and, in the absence of any treatment, palliative care remains the only option. The search for possible treatments remains imperative, yet current approaches are thwarted by the difficulty in delivering drugs or enzymes efficiently to the main site of pathology, the nervous system. The prospect of gene therapy for these diseases is appealing in that if the therapeutic protein could be targeted to the appropriate cell types, long-term expression may be achieved. Even if it may not be possible to target all diseased cells or the correct cell type, some lysosomal storage diseases would probably benefit from cross-correction, where the protein was secreted by one cell to be imported by its untransduced neighbours.

In selected cases, gene transfer to the adult nervous system has been successful. In one study, this was achieved using intramuscular injection of rabies pseudotyped lentivirus vectors, which permitted retrograde transport to spinal motor neurons where expression of the VEGF (vascular endothelial growth factor) transgene improved life expectancy in a mouse model of amyotrophic lateral sclerosis [2]. In other adult studies, direct administration to the CNS (central nervous system) has been the mode of delivery. For example, in a non-human primate model of Parkinson’s disease, intra-striatal injection of AAV (adeno-associated virus) vector carrying aromatic l-amino acid decarboxylase cDNA, improved clinical outcome for up to 8 years [3]. In children suffering from late infantile neuronal ceroid lipofuscinosis, AAV vector was injected intraparenchymally at six sites [4], but the interpretation of seemingly promising results was complicated by the difficulty of including appropriate controls. The most prominent and encouraging studies to date arise from three separate groups who have performed subretinal injection of AAV vectors to deliver the RPE65 cDNA to the subretinal space of patients with a form of retinal dystrophy called Leber’s congenital amaurosis [5–7]. Despite these promising studies where local or targeted delivery may be sufficient to treat localized and specific pathology, gene transfer to the adult CNS is insufficient when more global expression is required. Widespread gene delivery to the nervous system is feasible using fetal and neonatal delivery and may offer several advantages.

There are three fundamental advantages to fetal gene transfer that are also pertinent to neonatal gene transfer, at least in mouse models of disease. These advantages have been reviewed more thoroughly elsewhere [8–10], so will be discussed only briefly in the present paper. First, whereas transfer and expression of exogenous genes in the adult often results in immune elimination of foreign proteins, fetal or neonatal delivery leads to immune tolerance to the...
β-galactosidase expression was detected histochemically after gross dissection (left) and by light microscopy after paraffin wax processing (right) 1 month after fetal intraspinal injection of EIAV-lacZ.

expressed protein [11]. Several factors contribute to this favourable outcome. During the early stages of immune system development, extant proteins are perceived as ‘self’, and reactive immune cells are either eliminated or their activity is down-regulated. In addition, the fetal immune system is unlikely to have encountered the archetypal virus from which a given vector has been derived and therefore pre-existing anti-vector immunity is unlikely to arise. Secondly, at this age, there is a relative abundance of stem cells and actively dividing progenitors which can be efficiently transduced when exposed to the high concentration of vector that can be achieved in the relatively smaller fetus or newborn, and this is especially relevant for vectors that transduce dividing cells most efficiently. Moreover, transduction of progenitors by vectors which integrate into the host genome can result, in theory, in permanent gene expression in that tissue. Thirdly, for gene therapy, irreversible pathological changes may have already occurred by the time adult gene therapy is attempted, and a fetal approach may therefore prevent the subsequent onset of disease.

**Gene transfer as a potential therapy**

For most monogenic diseases, vector choice historically has broadly been determined by the technological advancement of each system at the time such as availability and ease of vector production. There has been a clear move towards high-efficiency systems containing minimal viral elements. The first studies showing gene delivery to the fetal brain were performed in the early 1980s. When Jaenisch and colleagues used a replication-competent retrovirus combined with a second modified retroviral vector containing the *Escherichia coli* gpt gene for injection into the mid-gestation mouse embryo, they observed transduction of several tissues, including the brain [12]. In this study, they already acknowledged the potential of this technology to correct genetic defects in somatic tissues. Shen and colleagues performed a series of studies investigating adenovirus vectors for gene transfer to the rat CNS, first by intra-amniotic injection at mid-gestation (12 dpc, where dpc is days post-coitus; gestation in mice and rats is approx. 20 days) [13] and then, more targeted, by injection into the lateral ventricle at late gestation (14 and 17 dpc) [14]. The late gestation injection, in particular, led to long-term expression for up to 10 months after injection, albeit at diminishing levels. Moreover, they were able to deliver and express β-glucuronidase cDNA in the CNS of a mouse model for mucopolysaccharidosis type VII, a lysosomal storage disease in which this enzyme is defective. Importantly, pathological lysosomal storage in neurons and glia was prevented for up to 4 months after injection [14]. These studies also illustrated the minimal immune response to fetal gene transfer even though AAV vectors are highly immunogenic.

The native tropism of HSV (herpes simplex virus) for neurons and its ability to deliver large genetic constructs approaching 130 kb has provided the rationale for studies using HSV-based vector systems. By using early-gene transfer, the immunogenicity of HSV vectors has been avoided, and the efficiency of this vector to infect dividing cells has been exploited [15,16]. To prolong the duration of expression, the authors incorporated the sleeping beauty transposon into the HSV amplicon to mediate integration into the host genome and achieved expression up to at least 3 months following injection.

A subset of retrovirus-derived vectors, the lentivirus vectors, are efficient at infecting non-dividing cells. We have observed transduction of multiple dorsal root ganglia and efferent nerves following intrathecal injection of an EIAV (equine infectious anaemia virus) lentivirus vector into fetal mice at 16 dpc (Figure 1). Recently, we have used potentially safer NILV (non-integrating lentivirus) vectors for fetal gene transfer. By using HIV lentivirus displaying different envelope glycoproteins (pseudotyping), we were able to modify their cellular tropism. The most commonly used
pseudotype, VSV-G (vesicular stomatitis virus glycoprotein), resulted in the strongest and most widespread transduction of neurons throughout the cortex, particularly layer V [17].

Possibly the most promising vector class for therapeutic intervention of the fetal or neonatal CNS and PNS (peripheral nervous system) is that of AAV. Credit should be given to Passini and Wolfe [18] for their early observation that an AAV serotype 2 vector efficiently delivered marker genes to newborn mice following injection into the lateral ventricle. In their study, excellent gene expression was observed in various CNS regions, including the dentate gyrus and inferior and superior colliculus, but very little expression was found in the ependyma or striatum. By switching to the AAV1 serotype, they achieved more widespread CNS transduction and were then able to use this vector to extend life expectancy in a mouse model of mucopolysaccharidosis type VII [19]. This study, which resulted in bilateral transduction and global cerebral enzyme activity within the brain, was the first to show substantial improvement in lifespan using fetal gene therapy to treat a lethal neurological condition. A second study, which emphasizes the potential of fetal gene therapy for prevention of CNS disease, was performed by Bennett and colleagues [20]. By injecting an AAV vector into the subretinal space of fetal mice (14 dpc) to deliver RPE65 cDNA, they were able to prevent blindness in the injected eye, in this mouse model of inherited blindness [20].

Using an equally precise injection technique, others have compared the efficiency of a range of AAV pseudotypes and lentivirus vectors for treatment of inherited deafness. In this study, injection by glass micropipette into the otocyst of mid-gestation mouse embryos resulted in long-term transduction in inner and outer hair cells. Hearing tests revealed no ototoxicity from any of the seven AAV pseudotypes tested, whereas there was mild hearing loss following administration of the VSV-G lentivirus vector [21].

Of the numerous AAV pseudotypes that are now available, AAV2/9 has recently stood out as one with astonishing ability to transduce cells of the nervous system. Crucially, this has been achieved by injection not via the intracranial route, but by the intravenous route in neonatal mice [22,23], cats [23] and non-human primates [24]. Foust et al. [24] have extended these studies by incorporating SMN (survival motor neuron) cDNA into this vector. Neonatal intravenous injection of this vector into the corresponding mouse model of spinal muscular atrophy resulted in an unprecedented improvement in survival and motor function [24]. The high transduction efficiency of this vector in the CNS is determined by its ability to cross the blood–brain barrier which has been proposed to occur by receptor-mediated events [25]. From a translational perspective, direct vector administration into the fetal brain or ventricles for fetal gene therapy of inherited neurological disease is unappealing. There are technical difficulties in injecting the fetal brain through the skull using minimally invasive injection techniques, although this has been achieved in non-human primate [26] and sheep (A.L. David, unpublished work) under ultrasound guidance. In contrast, ultrasound-guided access to the human fetal circulation is commonly used for fetal blood sampling and transfusions in clinical practice, with minimal fetal loss rate or complications [27]. Therefore the ability of AAV2/9 to cross the blood–brain barrier and transduce nervous tissue after systemic delivery holds intriguing potential for the treatment of fetal CNS pathology.

**Gene transfer as a study tool**

*In utero* gene transfer to the nervous system, particularly in rodent models, has developed along two increasingly divergent streams. In one stream are those researchers endeavouring to comprehensively transduce the CNS and PNS, their goal being to treat early-onset lethal diseases with gene therapy. The second stream consists of neurologists aiming to transduce highly selective cell populations as a tool for studying neurophysiology, neuropathology and neurological development.

A substantial body of work has accumulated, particularly over the last decade, in using gene-transfer technology as a means to study neurological development and function. In the late 1980s, three groups used retroviral vector technology to mark cell populations in the developing mouse fetus [28–30]. In a particularly elegant study in 1992, Walsh and Cepko [28] synthesized a library of retroviral vectors containing a series of unique genetic tags and the lacZ gene, which encodes the β-galactosidase marker protein. These vectors were injected into fetal rat brains at early (15 dpc) and later (17 dpc) time points in cortical neurogenesis, which continues until birth at 21 or 22 days of gestation. By analysing the distribution of each clone, they were able to demonstrate that specification of cortical areas occurs after neurogenesis. In a more recent study using ultrasound guidance to deliver a lentivirus vector into the amniotic fluid of the fetal mouse at 8 dpc, Endo et al. [31] were able to show transduction of ocular stem cells, specifically in the cornea, lens and retina.

In contrast with the application of fetal gene transfer as a potential therapy, vector choice has shifted over the last 10 years away from viral vectors to the delivery of naked DNA by electroporation to the murine nervous system [32]. Genetic labelling and tracking has been achieved in structures such as the marginal zone [32], dentate gyrus [33], amygdala [34], ventral metencephalon [35] and retinal ganglia [36]. Although electroporation is unlikely to have translational potential, it is does have advantages for gene transfer as a study tool. Whereas viral vectors are generally limited in the size of genetic construct that they can carry (e.g. retrovirus vectors can deliver no more than approx. 8 kb), purified DNA or RNA delivered by electroporation does not suffer the same size constraints. Therefore larger pieces of genetic material, containing one or more genes and more complete regulatory elements, may be delivered.

The technique has been used to co-deliver a GFP (green fluorescent protein) expression construct and a short hairpin RNA to transiently knock down DISC1 expression in normal mice. This resulted in adult behavioural deficits associated with disturbed cortical neurocircuitry after puberty [37]. This experiment epitomizes the application of this method...
to provide tissue- and developmental-stage-specific somatic transgenesis for the generation of new disease models.

In a highly elegant study, Brigande and colleagues delivered naked DNA for expression of AtoH1, a transcription factor required for auditory hair cell development, to the fetal murine cochlear [38]. This resulted in the production of functional sensory cells and demonstrated that this approach might be useful for studying auditory pathophysiology and also serve as a pathfinder for developing gene therapy for deafness [38].

Summary

At least in the near term, neonatal gene therapy would probably be preferred over fetal intervention. The above-mentioned correction of spinal muscular atrophy [24], alongside two similarly encouraging studies [39,40], illustrate the utility of neonatal gene therapy. Nevertheless, we have observed in mice that intravenous injection into fetuses and newborn animals results in starkly contrasting patterns of cell expression [41], which may dictate efficacy of gene therapy in different disease models. Moreover, there is evidence that, in some lysosomal storage diseases, such as acute neuronopathic Gaucher disease, pathological events are already occurring in utero [42]. Therefore pre-clinical studies using in utero gene transfer should be considered on a disease-by-disease basis, and it may be turn out that, where postnatal gene therapy is found wanting, fetal gene therapy may deliver.

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