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ADVANCES AND CHALLENGES IN PKU

Long-term correction of murine phenylketonuria by viral gene transfer: liver versus muscle

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Abstract Current therapy for phenylketonuria (PKU) consists of life-long dietary restriction of phenylalanine (Phe), which presents problems of adherence for patients. Alternative therapies under investigation include, among others, the use of gene therapy to provide copies of wild-type, non-mutant, phenylalanine hydroxylase (PAH) enzyme. Expression of PAH in both liver (the usual metabolic source of this enzyme) and skeletal muscle is under investigation. Liver gene therapy, using a viral vector based on the adeno-associated viruses (AAVs), provided effective clearance of serum Phe that was sustained for 1 year in some mice. In order for PAH expression to be effective in skeletal muscle, the essential metabolic cofactor, tetrahydrobiopterin (BH_4), must also be provided, either by supplementation or gene therapy. Both these approaches were effective. When transgenic PKU mice that constitutively expressed PAH in muscle were given intraperitoneal supplementation with BH_4, this produced (transient) effective clearance of Phe to normal levels. In addition, use of an AAV vector containing the genes for PAH, and for two key synthetic enzymes for BH_4, provided substantial and long-lasting correction (more than 1 year) of blood Phe levels when injected into skeletal muscle of PKU mice. These two strategies provide promising treatment alternatives for the management of PKU in patients.

Abbreviations

AAV	adeno-associated virus
BH_4	tetrahydrobiopterin
CMV	cytomegalovirus
EC	Enzyme Commission
ENU	*N*-ethyl-*N*-nitrosourea
GTP	guanosine triphosphate
GTPCH	guanosine triphosphate cyclohydrolase I
ITR	inverted terminal repeat
OMIM	Online Mendelian Inheritance in Man database
PAH	phenylalanine hydroxylase
Phe	phenylalanine
PKU	phenylketonuria
PTPS	6-pyruvoyl-tetrahydropterin synthase
rAAV	recombinant adeno-associated virus
SR	sepiapterin reductase
WPRE	woodchuck hepatitis virus post-transcriptional element

Introduction

Current treatments for phenylketonuria (PKU; OMIM 262600) consist of life-long dietary restriction of phenylalanine (Phe), with or without supplementation by amino acids, or, for patients responsive to this therapy, treatment with tetrahydrobiopterin (BH_4). Life-long dietary control of Phe intake is expensive, unpalatable, and difficult for patients to adhere to, as it severely restricts their lifestyle. It also presents additional problems for pregnant women, such as the fact that before and during pregnancy they must strictly maintain a low Phe diet to prevent foetal damage (the so-called maternal PKU syndrome). Alternative therapies are, therefore, being sought

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for this condition. Research into PKU has the advantage that treatment efficacy is easily measured (by the monitoring of plasma levels of Phe) and, ethically, adult patients who do not have cognitive problems can give informed consent to experimental therapies. One such treatment under investigation is gene therapy for the provision of functioning phenylalanine hydroxylase (PAH; EC 1.14.16.1) via viral vectors that are expressed in either liver or muscle. These two strategies for gene therapy in PKU are discussed below.

The PKU mouse

The mouse model used for testing gene therapy has the *Pah*^{enu2} ('PKU') mouse allele bred into the C57Bl/6 background (Ding et al. 2006; Shedlovsky et al. 1993). This strain was originally produced by chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU), which produced a missense mutation in the gene for PAH in exon 7 (F263S), a common site for mutations in human PAH. The gene is transcribed to mRNA and the mutated PAH protein is expressed, but this mutant protein has no enzymatic activity. This results in hyperphenylalaninaemia in the mice and a PKU-like syndrome that includes slow growth, hypopigmentation and maternal PKU syndrome. In wild-type mice, Phe concentrations are less than 100 μM, whereas those in PKU mice are between 1,500 μM and 2,500 μM (concentrations are slightly higher in female mice than in male mice). Phe concentrations can be determined by collection of a blood spot from the tail vein on a Guthrie card for tandem mass spectrometry.

Adeno-associated virus as a gene-delivery vector

Early research showed that liver-mediated gene therapy using, for instance, an adenovirus vector was able to produce a transient reversal of hypopigmentation in PKU mice (Nagasaki et al. 1999). However, this response was limited by an immune reaction to the vector. The adeno-associated viruses (AAVs) are non-pathogenic viruses, and as such, they appear to be minimally immunogenic (there may be pre-existing neutralizing antibodies for the commonly used serotype 2) and non-inflammatory. They have the potential to mediate long-term transgene expression in both dividing and non-dividing cells. To date, at least 14 serotypes of AAV, and more than 100 variants with different transduction profiles, have been described (Wu et al. 2006). The main disadvantages of these viruses as vectors for gene therapy are their relatively poor efficiency of transduction and organ specificity.

The wild-type AAV genome comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames, *rep* and *cap*. The *rep* reading frame contains four overlapping genes encoding Rep proteins that are required for the normal viral life cycle. The *cap* reading frame contains overlapping genes for three capsid proteins. The ITRs are the only cis-regulatory elements necessary for DNA replication and integration, so the *rep* and *cap* regions are deleted, and the therapeutic gene and a promoter are inserted between the ITRs. The resulting vector is thought to be episomally maintained, or it may be integrated at low frequency into the host-cell genome at a specific site on human chromosome 19; random integration is extremely low as a result of *rep* and *cap* deletion.

Liver gene therapy for phenylketonuria

For the construction of a vector for *PAH* gene transfer, the full-length mouse *Pah* complementary DNA (cDNA), a chicken β-actin (CBA) promoter for that sequence (cytomegalovirus enhancer–chicken β-actin promoter) and woodchuck hepatitis virus post-transcriptional element (WPRE) were inserted into a parent plasmid comprising AAV2 ITRs. The resulting plasmid pAAV2-PKU5 was co-transfected, together with two helper plasmids, into the human embryonic kidney cell line, 293 T. Recombinant (r) AAV2-PKU5/8 (serotype 8) vectors were then purified from harvested cells by caesium chloride (CsCl) gradient centrifugation, and the vector titre was determined by real-time quantitative polymerase chain reaction for WPRE (Ding et al. 2006)

PKU mice were given a single injection of the vector rAAV2-PKU5/8 (serotype 8) via the portal vein. Both male and female mice demonstrated phenotypic reversion by re-pigmentation of their coats within 2–8 weeks of treatment (Ding et al. 2006). Treated mice also showed dramatic reductions in plasma Phe concentrations to normal levels within 2 weeks, and this change was sustained in several mice for more than 1 year (Fig. 1). Whole-liver extracts from these mice showed PAH activity to be equivalent to that of the wild-type mice and showed no signs of toxicity, hepatocellular carcinoma or immunogenicity due to rAAV-vector transduction. Quantitative TaqMan® analysis of WPRE DNA showed only extremely low rates of transduction of other tissues by the PAH vector. Treatment with an AAV2 serotype 8 vector was also shown to be effective in managing maternal PKU in mice (Jung et al. 2008).

Relative efficacy of adeno-associated virus serotypes in producing liver transduction

Three AAV serotypes were tested for their effects in PKU mice, following intramuscular injection. The AAV2/1 (serotype 1) has a preferential tropism for skeletal muscle;

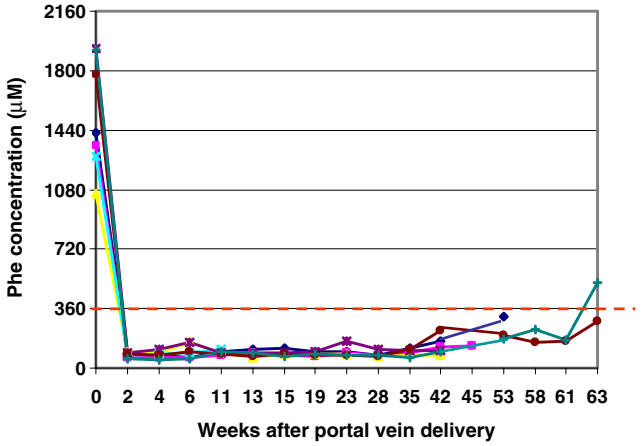

Fig. 1 Liver gene therapy with a serotype 8 adeno-associated virus (AAV) vector expressing PAH from the CBA promoter produced profound and long-lasting reductions in serum phenylalanine (Phe) concentrations in phenylketonuria (PKU) mice. Doses of 5×10^{12} vector genomes were administered by intraportal injection. The dashed line indicates the normal level of blood Phe (>0.36 mmol/l or 6 mg/dl) (for more details see also Ding et al. 2006)

AAV2/2 (serotype 2) transduces a wide range of tissue types, including muscle and liver; and AAV2/8 (serotype 8) shows selectivity for hepatocytes (Rebuffat et al. 2009) The serotype 1 vector, AAV2/1-PKU5, produced long-term effects on blood Phe levels in male mice (1 year; end of experiment) but less pronounced in female mice (approximately 30 weeks). This might be due to the androgen or sex-dependent influence on liver transduction by AAV-vector gene transfer, which is a known phenomenon (for references see Rebuffat et al. 2009). The serotype 2 vector, AAV2/2-PKU5, again provided benefits for male mice but had a limited effect on plasma Phe levels in female mice. By comparison, the serotype 8 vector, AAV2/8-PKU5, provided long-term effects on Phe in both male and female mice, but, similarly to the serotype 1 vector, only a transient correction was seen in the female mice (approx. 35 weeks). Two strategies were applied to increase or sustain transgene expression levels in AAV2-PKU5 treated female mice: administration of serotype 8 vector to mice previously treated with the AAV2/1 serotype led to prolonged therapeutic correction in female mice for a similar duration as was achieved in male mice. Alternatively, exogenous supplementation with BH_4 cofactor (by intraperitoneal injection) of AAV2-PKU5 serotype 2 or serotype 8 treated mice also led to a transient decrease in blood Phe levels.

Muscle gene therapy for phenylketonuria

Skeletal muscle comprises 30–40% of the mass of the human body, and much of it is easily accessible by percutaneous techniques (compared with access to the liver). Skeletal muscle is highly vascularized, and it contains both dividing and non-dividing cells with long half-lives, and stable episome expression. This tissue is therefore a good candidate for gene therapy. Besides PAH, its essential cofactor BH_4 is also not synthesized in muscle and must be supplied to muscle expressing PAH. Previously, it has been shown that transgenic PKU engineered to constitutively express PAH in muscle could clear serum Phe to normal levels upon intraperitoneal supplementation with BH_4 (Harding et al. 1998).

Production of BH_4 with phenylalanine hydroxylase in skeletal muscle

BH_4 is synthesized from guanosine triphosphate (GTP) in at least three enzymatic steps: GTP cyclohydrolase I (GTPCH) is the first enzyme in BH_4 biosynthesis that catalyses the

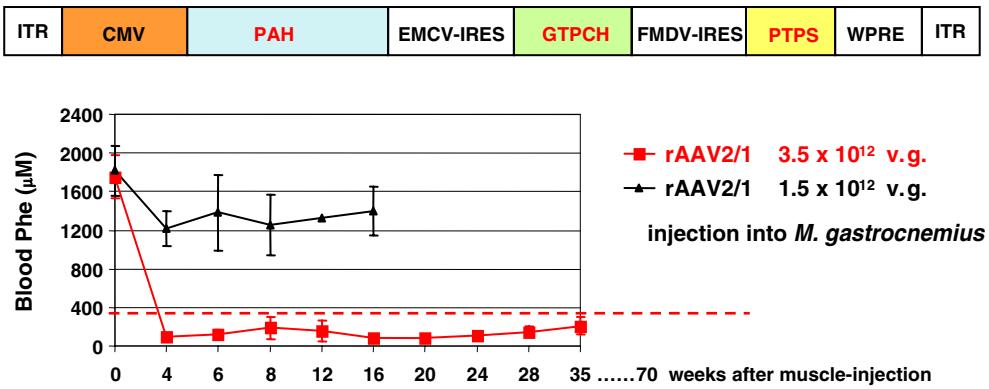

Fig. 2 Treatment with a three-gene vector, providing phenylalanine hydroxylase (PAH) and two synthetic enzymes for BH_4-corrected serum phenylalanine (Phe) levels in phenylketonuria (PKU) mice. The upper part shows the physical map of the recombinant AAV2 serotype 1 vector used, and the lower part shows the kinetic changes of blood Phe concentration in PKU mice after intramuscular administration of vector in two different doses. The dashed line indicates the normal level of blood Phe (>0.36 mmol/l or 6 mg/dl) (for more details see also Ding et al. 2008)

formation of 7,8-dihydroneopterin triphosphate from GTP. In the next step, 6-pyruvoyl-tetrahydropterin synthase (PTPS) catalyses the conversion of 7,8-dihydroneopterin triphosphate to 6-pyruvoyl-tetrahydropterin. Sepiapterin reductase (SR) then catalyses the final two-step reduction of 6-pyruvoyl-tetrahydropterin to BH_4. In wild-type mice, muscle levels of PAH, GTPCH, PTPS and total BH_4 are all present at non-detectable levels or are extremely low compared with those in the liver.(Thöny et al. 2000).

Transgenic PKU mice expressing both PAH and the BH_4-synthetic enzyme, GTPCH, in muscle tissue showed no BH_4 in muscle and, in the absence of its synthesis, accumulation of neopterin (Ding et al. 2008). Thus, the introduction of this enzyme was insufficient to provide BH_4 synthesis in muscle and reduce plasma Phe concentrations. Supplementation in these animals with a single dose of BH_4 produced a transient reduction in blood Phe (Ding et al. 2008). Similarly, when a two-gene recombinant AAV vector (serotype 1) expressing genes for PAH and for GTPCH from the (muscle specific) cytomegalovirus (CMV) promoter was injected into skeletal muscle in the PKU mouse, there was little effect on plasma Phe concentrations (Ding et al. 2008). However, a comparable three-gene vector containing genes for PAH, GTPCH and PTPS did provide substantial and long-lasting correction (more than 1 year) of blood Phe levels when injected into skeletal muscle of PKU mice (Fig. 2). In these mice, there were 20–107 vector genomes per diploid genome in foreleg muscle, the site of the AAV2/1 injection, and 1–2% PAH activity in muscle, compared with that in liver. There was some expression of the vector genome in liver, but very low or no expression in other body tissue, as would be expected upon injection of an AAV serotype 1 vector with a tropism for skeletal muscle tissue and expressing the transgenes from the muscle-specific CMV promoter (Ding et al. 2008).

Conclusions

Gene therapy using AAV vectors expressed in either the liver or skeletal muscle provides a promising treatment alternative for the management of PKU. Such therapy can provide normalization of serum levels of Phe, phenotypic reversion and management of maternal PKU in mice. In the future, optimization of this therapy should concentrate on the safety and longevity of the process. Thus, viral vectors, foreign or toxic bacterial DNA sequences and the use of endotoxins should be avoided. Use of an optimized liver or muscle promoter should allow the stable, long-term expression of genes, and life-long expression may require permanent integration of the exogenous gene into the host genome, or multiple applications of the therapy.

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