Current research on ORGANIC ACIDEMIAS

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The Organic Acidemia Association is proud to present the first edition of *Current Research on Organic Acidemias,* an educational publication designed to highlight and promote research studies on the organic acidemias.

We need to attract more graduate students, medical students and young physician scientists to the field of inborn errors of metabolism to hasten the development of better treatments and genetic cures for these disorders. Here, we present a broad cross-section of diseases, professionals and their unique ideas and approaches that tackle some hard questions: how do we best develop viable gene therapies for such disorders, and how do we unite the academic and clinical communities to generate successful clinical trials?

The current reality for patients with organic acidemias is tenuous. Expanded newborn screening methods can detect organic acidemias early in infancy and, in the United States, is being legislated by more and more states. Better dietary and clinical management of patients allows children born with these rare disorders to sometimes live longer than in the past, but it is impossible to measure the amount of ongoing damage a child constantly accumulates as he grows. At some point, the number of organ systems affected is too great for metabolic balance to be maintained, and even the mildest of cases can cascade into a toxic crisis that is often devastating to the patient and his family.

For the past twenty years, the Organic Acidemia Association has provided support to families dealing with the incredible life changes that come when a child with an organic acidemia is diagnosed. Our organization has touched the lives of hundreds of people who might otherwise have been left grasping in the dark for information and emotional support to help deal with such life transitions. Our mission would not be complete, however, without trying to inspire more young researchers to take a look at studying organic acidemias as possible career paths. Additionally, as more of our metabolic physicians are retiring we need more clinical professionals to take their places and to understand and adequately treat the long-term complications of these diseases until cures are found. Genetic cures for the organic acidemias urgently need to be developed, but more people need to study these disorders to make this a reality.

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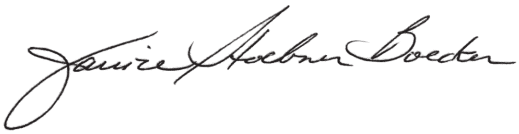

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THE ORGANIC ACIDEMIA ASSOCIATION
(OAA) provides information and support to parents and professionals dealing with a set of inborn errors of metabolism collectively called 'organic acidemias'. The OAA is a volunteer organization registered with the IRS as a 501c3 non-profit corporation. Donations to the OAA are tax-deductible. OAA publishes a newsletter three times a year, hosts an internet-based listserv for information exchange and maintains a website. These services are funded by donations from corporation and individual members. Annual membership donation of $25 (U.S) and $35 (international) plus $5 for the family roster is requested, but not required. Our 501(c)(3) non-profit status qualifies OAA for United Way donations through their write-in option. If there is a write-in option, just write "Organic Acidemia Association" in the blank line on your pledge card. Donations can also be made at OAA's website through the "PayPal" and the "Network for Good" option.

Progress in organic acidemias

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Although organic acidemias were described in the late 1960s, progress in understanding the pathophysiology has been relatively slow. As a result treatment is frequently not satisfactory and the outcome is often disappointing.

It is now clear that these disorders are heterogeneous. With the introduction of extended neonatal screening programmes, the spectrum of these disorders has continued to expand. In their paper, Drs. Vockley and Ensenauer (page 4), discuss a relatively common mild variant of isovaleric acidaemia, of which only one case was known until the introduction of these programmes. However, detection of isovaleric acidaemia enables treatment to start at an early stage and the outcome for those with severe disease has thereby improved.

By contrast the outcome after early diagnosis of methylmalonic (MMA) and propionic acidemia (PA) with conventional therapy is less satisfactory. These patients continue to have major complications including recurrent episodes of ketoacidosis, cognitive disabilities, pancreatitis, stroke-like episodes, cardiomyopathy, optic atrophy and renal failure. As a result the prognosis for patients with severe disease remains guarded. Although some patients do unexpectedly well, most do not. Unfortunately, as Dr. Tuchman discusses (page 13), we do not understand the cause of these complications, but it is hoped that long-term studies and detailed metabolic investigations in both patients and animal models will cast light on these problems.

Such studies are already under way in the USA, as described by Dr. Venditti and his colleagues (page 10), and in Europe led by investigators in Heidelberg and London. There is a particular need to develop treatment that is evidence-based, as almost none has been tested to current standards (Leonard, 2006).

Despite the difficulties, progress is being made in some areas. There is now a better understanding of glutaric aciduria type 1. An international cross-sectional study (Kölker *et al.*, 2006) combined with some individual clinical and pathological observations and laboratory work, including studies of a mouse model, has led to a better understanding of this disorder. It is now clear that the dicarboxylic acids that accumulate in this disease are neurotoxic and responsible for the acute neurological damage. These compounds are 'trapped' in the brain reaching high concentrations (Kölker *et al.*, 2004). The cross-sectional study confirmed the value of the low lysine diet, lysine being the main precursor of glutaric and 3-hydroxyglutaric acid. Restricting lysine intake reduces uptake by the brain and hence the accumulation of the toxic metabolites. Guidelines have now been published for the management of this disorder (Kölker *et al.*, 2007) and we hope that these guidelines, combined with early detection in neonatal screening programmes, will greatly improve the outcome of this devastating disorder.

The recent work on glutaric aciduria type 1 on the mechanisms of brain damage may have wider implications in related disorders including MMA and PA (Kölker *et al.*, 2006). This may help to reduce the burden of neurological disability. However, given the poor outcome of PA and MMA, alternatives to conventional dietary treatment have been explored. The role of liver and kidney transplantation for MMA is uncertain and, although widely discussed, both the indications and timing remains unclear (Barshes *et al.*, 2006, Kasahara *et al.*, 2006). It has to be recognised that the enzymes affected in most organic acidemias are expressed in almost all tissues, but liver transplantation only replaces them in that organ. Although this reduces the metabolic effects of the disease, it does not correct the enzyme deficiency in the brain. This seems to matter less in patients with PA than with MMA, who may have stroke-like episodes years after successful transplantation (Chakrapani *et al.*, 2002).

Better forms of treatment are urgently needed with the aim of correcting the deficiency in all tissues. Although the work on viral vectors for gene transfer discussed by Drs. Hofherr and Barry (page 20) is promising, many hurdles have yet to be overcome. Other methods of genetic manipulation hold out more immediate promise. As discussed by Dr. Kraus (page 17), chemical chaperones could be used in people with missense mutations to stabilize the mutant enzyme, correct misfolding and ensure that the enzyme reaches the intended site in the cell. This should increase the residual enzymatic activity sufficiently to ameliorate the disease, even if it does not cure it (Frustaci *et al.*, 2001; Ishii *et al.*, 2004). Several of these chemical chaperones are already in use in metabolic patients. For example, sodium phenylbutyrate is used as a nitrogen scavenger in organic acidaemias and urea cycle disorders.

Altering the activity of aberrant splicing also looks promising, as highlighted by Dr. Ugarte and her colleagues (page 24). Sodium phenylbutyrate can also be used to this end as well, as it is also a histone deacetylase inhibitor. As several of these compounds are already in clinical use, trials could be imminent.

Oligonucleotides may prove to be useful in correcting splice site mutations and another possible line of treatment is the correction of mutations *in situ*. Whilst some of these have promise for correcting monogenic diseases (for a brief review of both gene targeting and delivery, see Tachikawa and Briggs, 2006), there are many problems and a long way to go before they can be applied to those with organic acidaemias.

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Isovaleric acidemia: new aspects of genetic and phenotypic heterogeneity

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Isovaleric acidemia (IVA) is an autosomal recessive inborn error of leucine metabolism caused by a deficiency of the mitochondrial enzyme isovaleryl-CoA dehydrogenase (IVD) resulting in the accumulation of derivatives of isovaleryl-CoA. It was the first organic acidemia recognized in humans and can cause significant morbidity and mortality. Early diagnosis and treatment with a protein restricted diet and supplementation with carnitine and glycine are effective in promoting normal development in severely affected individuals. Both intra- and interfamilial variability have been recognized. Initially, two phenotypes with either an acute neonatal or a chronic intermittent presentation were described. More recently, a third group of individuals with mild biochemical abnormalities who can be asymptomatic have been identified through newborn screening of blood spots by tandem mass spectrometry. IVD is a flavoenzyme that catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA and transfers electrons to the electron transfer flavoprotein. Human IVD has been purified from tissue and recombinant sources and its biochemical and physical properties have been extensively studied. Molecular analysis of the IVD gene from patients with IVA has allowed characterization of different types of mutations in this gene. One missense mutation, 932C>T (A282V), is particularly common in patients identified through newborn screening with mild metabolite elevations and who have remained asymptomatic to date. This mutation leads to a partially active enzyme with altered catalytic properties; however, its effects on clinical outcome and the necessity of therapy are still unknown. A better understanding of the heterogeneity of this disease and the relevance of genotype/phenotype correlations to clinical management of patients are among the challenges remaining in the study of this disorder in the coming years.

Isovaleric acidemia (IVA) was the first of the organic acidemias to be described and its nature was elucidated due to a combination of astute clinical acumen and new technology. The two original patients were 4 and 21/2 years old, respectively, and had similar histories of recurrent episodes of vomiting and lethargy that resolved with supportive therapy including glucose infusions (Tanaka *et al.*, 1966; Budd *et al.*, 1967; Efron, 1967). A "smell specialist" suggested that the unusual odor associated with their acute episodes was likely a short chain fatty acid, and isovaleric acid was identified in patient plasma through the then novel approach of gas chromatography followed by mass spectrometry (Tanaka, 1990). Urine was subsequently shown to contain isovalerylglycine and 3-hydroxyisovaleric acid as well as other metabolites (Tanaka and Isselbacher, 1967; Tanaka *et al.*, 1968). Oxidation of labeled isovaleric acid by patient white blood cells was reduced compared to control cells, and a defect of leucine metabolism was postulated (Shih *et al.*, 1973; Rhead and Tanaka, 1980; Yoshida *et al.*, 1985; Hyman and Tanaka, 1986). It must be remembered that until the characterization of these patients, the oxidation of isovaleryl-CoA was assumed to be catalyzed by the newly identified short chain acyl-CoA dehydrogenase (Crane *et al.*, 1956; Engel and Massey, 1971; McKean *et al.*, 1979; Frerman *et al.*, 1980). Over the next 20 years IVD was separated from the other acyl-CoA dehydrogenases, purified, cloned, and the molecular nature of the defects responsible for loss of IVD activity elucidated. The use of gas chromatography/mass spectrometry (GC-MS) ultimately became the mainstay of the identification and routine clinical diagnosis of a new class of inborn errors of metabolism resulting in the abnormal accumulation of organic acids in urine, and it remains a valuable tool for biochemical geneticists today (Tanaka *et al.*, 1980; Burke *et al.*, 1983). The more recent application of tandem mass spectrometry (MS/MS) for analysis of the acylcarnitine profile of blood spots from newborn screening filter paper cards has allowed a significant expansion of the recognition of mildly affected and potentially asymptomatic individuals with IVD deficiency through newborn screening (Ensenauer *et al.*, 2004).

Disease spectrum

Early literature on IVA, an autosomal recessive disorder, emphasized two apparent phenotypes (Tanaka, 1990). The first was an acute, neonatal presentation with patients becoming symptomatic within the first 2 weeks of life (Tanaka *et al.*, 1966; Budd *et al.*, 1967; Efron, 1967; Lott *et al.*, 1972; Levy *et al.*, 1973; Elsas and Naglak, 1988). Patients appeared initially well, then developed vomiting and lethargy, progressing to coma. The second group presented with relatively non-specific failure to thrive and/or developmental delay (chronic intermittent presentation) (Levy *et al.*, 1973; Shih *et al.*, 1984; Berry *et al.*, 1988; Elsas and Naglak, 1988; Mehta *et al.*, 1996). Patients who survived an early acute presentation subsequently were indistinguishable from those with the chronic phenotype, and both groups of patients were prone to intermittent acute episodes of decompensation with minor illnesses (Tanaka, 1990). In reality it is now apparent that patients can fall anywhere on the spectrum of acute to chronic presentation and that there is probably little predictive value to the initial presentation. Moreover, with the application of MS/MS in newborn screening, potentially asymptomatic patients with one recurring IVD gene mutation and a mild biochemical phenotype are being identified in increasing numbers, representing an additional phenotype of IVA (Ensenauer *et al.*, 2004). This type may represent a biochemical phenotype only without the expression of any clinical symptoms (such as in benign hyperphenylalaninemia) and, therefore, needs to be differentiated from the classic forms of IVA. For practical purposes, we suggest classifying patients with IVA as "metabolically severe" and "metabolically mild or intermediate", giving consideration to the broader spectrum of IVA detectable by newborn screening.

Clinical presentation

Nearly all published clinical information on patients with IVA is retrospective (Sweetman and Williams, 2001), and thus the following discussion of clinical symptoms is limited to the classic presentations of IVA prior to newborn screening, that is, manifestation in the neonatal period versus later in childhood.

In one summary of 37 patients compiled from different publications, 28 presented in the first 2 weeks of life, 7 between 2 weeks and 1 year of age, and the remaining 2 after 1 year of age (Tanaka, 1990). Sixteen of the patients were deceased, and of those still alive, seven were reported to have mild to moderate mental retardation.

Neonatal symptoms are non-specific and include poor feeding, vomiting, decreased level of consciousness, and seizures (Tanaka *et al.*, 1966; Budd *et al.*, 1967; Efron, 1967; Lott *et al.*, 1972; Levy *et al.*, 1973; Spirer *et al.*, 1975; Elsas and Naglak, 1988). Infants may develop hypothermia and appear to be dehydrated. A characteristic smell of "dirty socks" may be present when the patient is acutely sick though, unlike other organic acidemias, the urine has no odor since the unconjugated isovaleric acid responsible for the odor is not excreted in urine in appreciable quantity (Tanaka *et al.*, 1966; Tanaka, 1990). The odor may be best appreciated in body sweat or cerumen from the ear. Acidosis with an unexplained anion gap is characteristic, and hyperammonemia, hyper- or hypoglycemia and hypocalcemia may be present (Tanaka *et al.*, 1966; Budd *et al.*, 1967; Lott *et al.*, 1972; Levy *et al.*, 1973; Yoshino *et al.*, 1982; Mendiola *et al.*, 1984; Tanaka, 1990; Worthen *et al.*, 1994). Secondary hyperammonemia is presumed to be due to inhibition of *N*-acetylglutamate synthetase by isovaleryl-CoA and/or intracellular depletion of acetyl-CoA leading to reduced *N*-acetylglutamate synthesis and impairment of the urea cycle (Coude *et al.*, 1979; Stewart and Walser, 1980). Pancytopenia, as well as isolated neutropenia and thrombocytopenia, can occur due to bone marrow suppression (Kelleher *et al.*, 1980). Left untreated, patients may progress to coma and death often due to cerebral edema or hemorrhage (Fischer *et al.*, 1981). Overall, the clinical picture overlaps other organic acidemias including the β-oxidation defects as well as the urea cycle disorders and other primary causes of hyperammonemia, all of which must, therefore, be considered in the differential diagnosis. Patients who survive a neonatal crisis may be clinically indistinguishable from children diagnosed later in life (Tanaka, 1990).

Children diagnosed outside the newborn period may present with more chronic, relatively non-specific findings of failure to thrive and/or developmental delay or mental retardation (Tanaka, 1990). Minus the "sweaty feet odor" of isovaleric acid, which is not present when a patient is otherwise well, there is little to suggest a specific diagnosis in these children and thus, it must be considered in all patients with this clinical picture. They also are at risk of episodes of acute acidosis and metabolic decompensation, usually due to intercurrent illnesses or other physiologic stress including fasting (Berry *et al.*, 1988; Tanaka, 1990). Acute episodes may be misdiagnosed as diabetic ketoacidosis due to hyperglycemia, acidosis and the apparent presence of blood and urinary ketones (Attia *et al.*, 1996). Acute pancreatitis, myeloproliferative syndrome, Fanconi syndrome, and cardiac arrhythmias have been reported (Arnold *et al.*, 1986; Kahler *et al.*, 1994; Weinberg *et al.*, 1997; Gilbert-Barness and Barness, 1999); abnormalities of the globus pallidus can be seen (Sogut *et al.*, 2004). Age of crises can be variable as highlighted by the report of a well-controlled 18-year-old man with IVA who developed acute nausea, vomiting, and mental status changes during basic training camp for the United States Air Force (Feinstein and O'Brien, 2003).

There have been reports of successful pregnancies in women with IVA resulting in apparently well infants (Shih *et al.*, 1984; Spinty *et al.*, 2002).

Biochemical diagonosis and follow-up

The majority of patients with IVA today are diagnosed pre-symptomatically through newborn screening by use of MS/MS, which reveals elevations of the marker metabolite C5 acylcarnitine in dried blood spots. Because C5 acylcarnitine represents a mixture of isomers (isovalerylcarnitine, 2-methylbutyrylcarnitine, and pivaloyl-carnitine), further diagnostic evaluation is required (Table 1). Elevations of 2-methylbutyrylcarnitine are seen in patients with 2-methylbutyrylglycinuria caused by a deficiency of short/ branched-chain acyl-CoA dehydrogenase (SBCAD), an inborn error of isoleucine catabolism (Andresen *et al.*, 2000; Gibson *et al.*, 2000), whereas pivaloylcarnitine is derived from pivalic acid, a component of several antibiotics (Abdenur *et al.*, 1998). Isovaleryl-CoA intermediates can also be seen in deficiencies of the electron transfer flavoprotein (ETF) and its dehydrogenase (glutaric aciduria type 2).

A long list of other isovaleryl-CoA derived metabolites has been reported in blood and urine from patients with IVA and can assist in confirmation of the disorder (Lehnert, 1981a,b, 1983; Lehnert and Niederhoff, 1981; Burke *et al.*, 1983; Dorland *et al.*, 1983; Hine and Tanaka, 1984; Poorthuis *et al.*, 1993; Loots *et al.*, 2005). Isovaleryl conjugates of multiple amino acids have also been detected in urine, as have free 3- and 4-hydroxyisovaleric acids (Tanaka *et al.*, 1968; Lehnert and Niederhoff, 1981; Loots *et al.*, 2005). Free isovaleric acid in blood during episodes of acute metabolic decompensation can reach several hundred times normal values but is not readily seen in blood and urine due to its rapid conjugation to other compounds. Thus, isovalerylcarnitine and isovalerylglycine are the hallmarks of this disorder in plasma and urine, respectively, and are elevated regardless of a patient's metabolic condition. Quantification of both conjugates has suggested a correlation of the metabolite concentrations with genotype, differentiating between groups of patients with a metabolically severe phenotype associated with heterogeneous *IVD* gene mutations and patients with a metabolically mild or intermediate phenotype associated with one recurring mutation (Ensenauer *et al.*, 2004). Disease-specific metabolites also accumulate in amniotic fluid during pregnancy with an affected fetus and provide the opportunity for prenatal diagnosis (Jakobs *et al.*, 1984; Hine *et al.*, 1986; Shigematsu *et al.*, 1991).

Several direct and indirect methods to assay IVD activity have been published and, in addition to molecular genetic analysis, can be used to confirm a diagnosis of IVA (Shih *et al.*, 1973; Rhead and Tanaka, 1980; Yoshida *et al.*, 1985; Hyman and Tanaka, 1986; Vockley *et al.*, 1991;

Table 1. Initial diagnostic evaluation for isovaleric acidemia[a]

Test	Determination of
Urine organic acid analysis	Multiple abnormal metabolites; isovalerylglycine concentration
Plasma acylcarnitine analysis	Isovalerylcarnitine concentration
Plasma carnitine analysis	Free carnitine concentration
Molecular genetic analysis	Common 932C>T (A282V) IVD gene mutation associated with a mild biochemical phenotype; otherwise heterogeneous mutations
Enzymatic analysis, optional (fibroblasts, lymphocytes)	Residual enzyme activity

[a]Evaluation following abnormal newborn screening with an elevated C5 acylcarnitine concentration

Mohsen *et al.*, 1998; Tajima *et al.*, 2005). Fibroblasts, lymphocytes, and amniocytes all have measurable amounts of IVD activity and serve as ready sources of tissue for this purpose (Vockley *et al.*, 1991; Kleijer *et al.*, 1995; Mohsen *et al.*, 1998; Ensenauer *et al.*, 2004). While significant residual activity blunts the level of abnormal metabolites, correlation between clinical presentation and enzyme activity has been poor (Ikeda *et al.*, 1985b; Hyman and Tanaka, 1986; Vockley *et al.*, 1991).

Regarding routine follow-up visits, there is no established laboratory marker for monitoring therapeutic control or disease state. Weight gain, growth and development should be age-appropriate and thus, body measurements are key parameters to follow on a routine basis. Specifically, protein malnutrition must be avoided if the patient is protein restricted. Analysis of amino acids, albumin, and prealbumin in plasma is recommended to monitor this. Plasma levels of leucine are not elevated in IVA, even if untreated, due to the irreversible oxidative decarboxylation earlier in the leucine degradation pathway (Sweetman and Williams, 2001). Plasma free carnitine concentrations may be helpful for determining necessity and monitoring of carnitine supplementation.

Molecular findings

IVD is an intramitochondrial homotetrameric flavoenzyme that catalyzes the α,β-dehydrogenation of isovaleryl-CoA resulting in 3-methylcrotonyl-CoA (Fig. 1) and transfers the reducing equivalents to ETF (Ikeda *et al.*, 1983; Ikeda and Tanaka, 1988; Mohsen and Vockley, 1995). It is encoded in the nuclear genome as a precursor protein, but is active in the mitochondrial matrix (Vockley *et al.*, 1991; Ikeda *et al.*, 1987; Parimoo and Tanaka, 1993). Following transcription in the nucleus and translation in the cytoplasm, it is held in a partially folded state by chaperonin proteins, targeted to mitochondria by a characteristic amino terminal peptide sequence and imported into mitochondria. The target peptide is cleaved in the mitochondrial matrix, folding of the monomer is completed, and the final active tetramer is assembled. The FAD cofactor is probably added after import into the mitochondria, but the exact timing and mechanism of this reaction is poorly characterized (Nagao and Tanaka, 1992). The reaction catalyzed by IVD is initiated upon acyl-CoA substrate binding. In the reductive half reaction, the formation of a hydrogen bond between the acyl carbonyl oxygen and the 2′-hydroxyl hydrogen of the FAD ribityl moiety is crucial for activation of the acyl moiety (Ghisla *et al.*, 1992; Nishina *et al.*, 1995; Miura *et al.*, 1996). The C_α and C_β hydrogens of the acyl moiety are then removed in concert as a proton and a hydride, respectively, forming a stable charge-transfer complex. ETF, considered a second substrate, extracts the reducing equivalents from the charge-transfer complex in the oxidative half-reaction during which electrons are transferred to the latter and the enoyl-CoA product is released (Gorelick *et al.*, 1985; Ikeda *et al.*, 1985a; Ghisla and Massey, 1989). The molecular mechanism of the oxidative half-reaction and the order of binding of the substrates have not been elucidated.

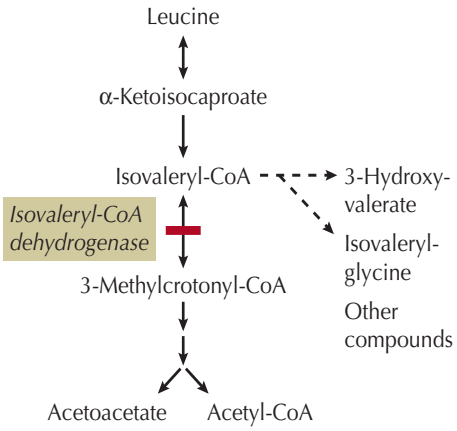

Figure 1. The catabolic pathway of leucine. Isovaleryl-CoA dehydrogenase catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA. Enzyme deficiency results in the accumulation of isovaleryl-CoA derivatives.

Molecular cloning of the *IVD* gene showed it to be located on chromosome 15q14-15, consisting of 12 exons that span 15 kb of genomic DNA (Parimoo and Tanaka, 1993). Molecular analysis of the *IVD* gene in patients with symptomatic IVA has identified numerous point mutations in the protein coding region that lead to the production of an inactive or unstable protein (Fig. 2) (Ikeda *et al.*, 1985b; Vockley *et al.*, 1991, 1992a,b, 2000; Mohsen *et al.*, 1998). Some of these have been shown to cause only a mild alteration in enzyme function, correlating to some extent with a mild clinical phenotype in patients (Mohsen *et al.*, 1998; Nasser *et al.*, 2004). A significant proportion of the mutant alleles lead to abnormal splicing of the *IVD* RNA and subsequent complete lack of IVD protein (Vockley *et al.*, 1991, 2000). Studies of the bioprocessing of IVD protein in fibroblasts from patients with IVA have reflected the effects of these mutations but have not provided insight into the clinical variability seen in the disorder (Ikeda *et al.*, 1985b; Vockley *et al.*, 1991). In general, genotype and phenotype have not been well correlated.

Newborn screening and isovaleric acidemia

With the advent of the use of MS/MS to screen newborn blood spots for acylcarnitine concentrations, most if not all patients with IVA should be identified as newborns prior to the development of symptoms. One unexpected finding to arise from newborn screening studies is the identification of individuals with only mild elevations of isovaleryl-CoA related metabolites in plasma and urine, orders of magnitude lower than in the classic forms of IVA, and apparently only partial reduction in IVD activity (Table 2) (Ensenauer *et al.*, 2004). Nearly half of the mutant IVD alleles sequenced from infants diagnosed by newborn screening have been found to contain a common recurring missense mutation (932C>T; A282V; Fig. 2) (Ensenauer *et al.*, 2004). This specific mutation was present in approximately two thirds of newborns in this cohort, mostly in a compound heterozygous fashion. All of the affected newborns carrying the common mutation have remained asymptomatic with

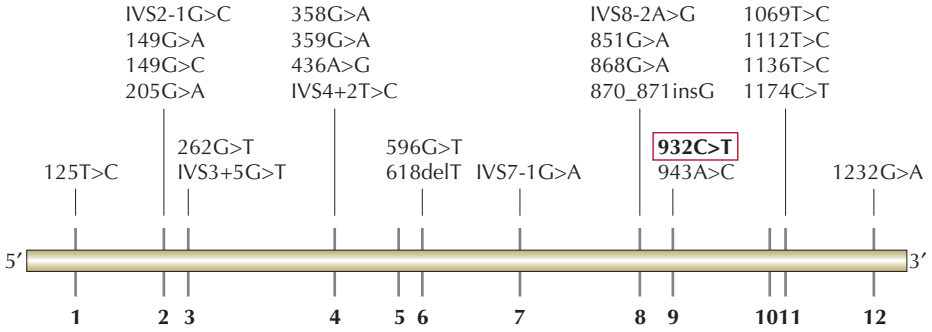

Figure 2. Molecular defects in the *IVD* gene on chromosome 15q14-15 (Mohsen *et al.,* 1998; Ensenauer *et al.,* 2004). The nucleotide change 932C>T (A282V) in exon 9 has been identified in a significant proportion of individuals diagnosed by newborn screening (Ensenauer *et al.,* 2004).

mild or no dietary protein restriction and carnitine supplementation if necessary over a maximum duration of follow-up of up to 5 years of age. Subsequently, asymptomatic siblings of patients identified through newborn screening (ages 3–11 years at the time of diagnosis) have been found to be homozygous or compound heterozygous for the same mutation with a similarly mild biochemical phenotype (Ensenauer *et al.*, 2004). They have remained without symptoms during episodes of febrile illnesses.

Prior to newborn screening, this mutation was identified only in a single patient with mild IVD deficiency originally evaluated for a tic disorder and slight developmental delay (Vockley *et al.*, 1992b; Mohsen *et al.*, 1998). The mutant A282V protein is stable in vitro but is kinetically impaired, exhibiting an increased K_m and a reduced catalytic efficiency, and has diminished thermal stability (Mohsen *et al.*, 1998; Nasser *et al.*, 2004). It is clear that the newborn screening patients who carry the common mutation either in a homozygous or compound heterozygous state and their sibs skew the spectrum of IVA with more than half of individuals representing a new mild phenotype and potentially remaining asymptomatic. This is an expansion of our view of the natural history of IVA prior to the newborn screening era and leads to significant implications for management and genetic counseling. As opposed to the classic forms of IVA, it is still uncertain whether individuals with the latter type have a disease, a risk of clinical manifestation, or simply express a clinically insignificant biochemical phenotype. While these individuals may have normal leucine homeostasis under physiological conditions, their risk of metabolic decompensation under stress conditions remains to be elucidated.

Therapy

There are three goals for therapy of IVA (Table 2). The first is prevention of metabolic decompensation by careful clinical observation of the patient regardless of the type of IVA. During times of metabolic stress (including illness and fasting) endogenous leucine from protein catabolism adds significantly to the production of isovaleryl-CoA (Collins *et al.*, 1987; Millington *et al.*, 1987; Pollitt, 1987). Achieving anabolism is the main therapeutic approach. Thus, sick day precautions for patients with IVA should include increased caloric intake in addition to decreased leucine intake. This is most easily accomplished with oral solutions containing simple sugars and leucine free metabolic formulae or powders. IV glucose infusions need to be added if oral intake is interrupted. Leucine intake should be decreased to approximately 50% of the patient's usual daily minimum, but returned to normal after 24 hr in order to promote protein anabolism. The second goal is long-term reduction of the production of isovaleryl-CoA from leucine catabolism through dietary manipulation (Lott *et al.*, 1972; Levy *et al.*, 1973; Berry *et al.*, 1988; Sweetman and Williams, 2001). Total protein and caloric intake must be adequate to support normal growth in children and maintain an anabolic state, and thus monitoring of weight, length, and head circumference is essential at follow-up. In many cases, it may be sufficient to moderately lower protein intake with natural foods to approximately 1.5 gm/kg per day. In patients with recurrent clinical symptoms, leucine restriction in excess of total natural protein may also be necessary (Sweetman and Williams, 2001). Natural protein necessary to reach the recommended age-appropriate daily requirement must then be provided with leucine-free amino acids. Because of the specific role of leucine in promoting protein synthesis, however, there is a potential for adverse side effects of rigorous leucine restriction including muscle wasting (Harris *et al.*, 2004). Acute episodes of metabolic decompensation can present with emesis, lethargy and signs of overwhelming acidosis. Under these circumstances, immediate hospitalization is required so that IV access can be established and glucose administered. Glucose infusion should be calculated to give at least 8 mg/kg per min with concomitant use of IV insulin if necessary to maintain euglycemia. Reintroduction of oral intake including catabolism-sparing levels of protein (0.5 gm/kg per day) with leucine should occur as soon as it can be tolerated, otherwise parenteral amino acids should be provided. If present, hyperammonemia will reverse with correction of the primary metabolic derangement; alternative ammonia conjugating agents such as sodium benzoate or phenylbutyrate are generally not indicated. The third goal of therapy in patients with IVA is to prevent the accumulation of toxic metabolites by shunting isovaleryl-CoA towards reactions that produce metabolites presumed to be non-toxic and that can readily be excreted. Recognition of isovalerylglycine in urine in the initial patients with IVA first led to the use of glycine to achieve this end (Krieger and Tanaka, 1976; Cohn *et al.*, 1978; Yudkoff *et al.*, 1978; Elsas and Naglak, 1988). Isovaleryl-CoA is enzymatically conjugated to glycine, a reaction that can be augmented by supplementation with exogenous glycine to supra-physiologic levels. Such supplementation prevents or reduces the accumulation of isovaleric acid in blood following a leucine load, and the length and severity of symptoms during intercurrent illnesses (Krieger and Tanaka, 1976; Yudkoff *et al.*, 1978; Shigematsu *et al.*, 1982). Doses of 150–600 mg/kg per day given orally and divided in three or four equal doses of body weight have been proposed, but the optimum dose has not been determined. Patients exhibit a dose sensitive increase in excretion of isovaleryglycine, but at least in one report an increase in the glycine dose from 300 to 600 mg/ kg/day of body weight led to a decrease in the excretion of isovalerylglycine, presumably due to inhibition of glycine-*N*-acylase by glycine (Elsas and Naglak, 1988). Thus, initial dosing in the range of 150–250 mg/kg per day is reasonable in patients with a metabolically severe type of IVA (Table 2). Concern has been raised about the potential for glycine toxicity, though no reports of such an occurrence have been published.

Table 2. Recommendations for Therapy in Isovaleric Acidemia

	Biochemical phenotype	
Therapy	**Metabolically mild or intermediate[a]**	**Metabolically severe[b]**
Prevention of metabolic crisis	Close clinical observation; promote anabolism during illness	
Diet	None	Protein restriction
Medication	Carnitine (30–50 mg/kg per day) if plasma free carnitine concentration is low	Carnitine (100 mg/kg per day)
	None	Glycine (150–250 mg/kg per day)

[a]Metabolically mild or intermediate: newborn screening blood spot C5 acylcarnitine concentration 0.8–6.0 mmol/L; urine isovalerylglycine concentration 15–195 mmol/mol creatinine.
[b]Metabolically severe: newborn screening blood spot C5 acylcarnitine concentration up to 21.7 mmol/L; urine isovalerylglycine concentration up to 3,300 mmol/mol creatinine.
Values derived from Ensenauer *et al.,* 2004.

The identification of isovalerylcarnitine in blood and urine along with the frequent observation of a secondary deficiency of free carnitine in patients with IVA has prompted treatment with carnitine. A dose of 100 mg/kg body weight per day has generally been used (TABLE 2) and has been shown to increase the excretion of isovalerylcarnitine in urine (Mayatepek *et al.*, 1991; Fries *et al.*, 1996). Combined therapy with carnitine and glycine has been shown to maximize the total excretion of isovaleryl-CoA conjugates, but the clinical benefit of combined versus single therapy has not been established through controlled studies (Fries *et al.*, 1996; Itoh *et al.*, 1996). The relative merits of the two therapies either singly or together in patients with more severe presentations including recurrent crises remains a matter of debate.

The necessity of any treatment for individuals diagnosed by newborn screening and carrying the common 932C>T (A282V) mutation is unclear. Specifically, the potential for metabolic decompensation under stress conditions remains to be elucidated. It appears reasonable to observe these individuals clinically, particularly when exposed to metabolic stressors such as febrile illnesses or fasting (e.g., when undergoing surgery). Additional recommendations include low-dose carnitine supplementation if the plasma free carnitine concentration is reduced (TABLE 2).

Summary

IVA was originally viewed as a relatively rare, life threatening inborn error of metabolism with both acute and chronic manifestations. Recent data from newborn screening studies and additional molecular and cellular laboratory investigations have revealed a far more heterogeneous condition with a potential for normal growth and development. Prospective long-term follow-up of newborns identified with IVA and clinical trials of carnitine and glycine therapy will be critical to optimization of outcome in these patients.

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Translational research approaches to study methylmalonic acidemia

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Our integrated approach to study methlymalonic acidemia and related disorders has been to define the natural history observed in patients using clinical research conducted at the National Institutes of Health Clinical Research Center and to investigate the disorder in the laboratory using metabolic, genetic and genomic approaches *(1)*. Our long-term objectives are to understand the pathophysiology of the complications in the patients, replicate the disorders in model organisms, and assess new therapeutic approaches in the laboratory and eventually, in patients.

The clinical foundation for understanding the phenotype of methymalonic acidemia derives from a series of reports published in the late 1960s (*2–4*). Oberholtzer *et al*. described two unrelated patients with methylmalonic acidemia (*2*). The first affected individual was initially suspected to have a renal tubular dysfunction syndrome. This patient had frequent attacks of dehydration and acidosis, and perished during a decompensation at age 21 months. On postmortem examination, fungal pneumonia was noted and a peculiar histopathological appearance of the kidneys was documented. Specifically, the kidneys were shrunken, and the tubules were diminutive and had increased interstitial tissue with a lymphocytic infiltration. The second child displayed a similar phenotype with respect to the acid-base instability. Classical analytical chemical methods were used to demonstrate that she produced large amounts of methylmalonic acid (MMA) in her urine, blood and cerebrospinal fluid (CSF). Of note, she had a CSF:plasma gradient, with concentrations of MMA equal to 1.55 mM in her plasma and 1.575 mM in her CSF. The authors also noted that the metabolic acidosis was only partly explained by the plasma MMA levels. A propensity toward ketosis was demonstrated, with an exquisite sensitivity to oral propionic acid. The child had problems with growth and motor skills in the early years, but when assessed at 5 years of age she had a normal IQ.

The same year Stokke *et al*. studied the third child born to a family that had two infants perish in the newborn period with overwhelming acidosis and coma (*3*). They also demonstrated that the patient produced MMA in enormous amounts. Whole-body metabolism was studied in the index case with C^{14}-valine and H^3-MMA. The patient did not respond to parenteral cobalamin but did demonstrate a clinical improvement with simple hyperalimentation consisting of elemental amino acids and glucose given intravenously, and fats and carbohydrates administered by nasogastric feeding, prior to perishing from an intercurrent infection. In the next year, a patient with a similar phenotype of intermittent ketoacidosis and severe methylmalonic aciduria was proven to respond to vitamin B12, firmly establishing a role for the vitamin in human intermediary metabolism (*4,5*).

The early studies on patients with methymalonic acidemia generated theories to explain the metabolic perturbations seen in the affected patients, demonstrated fundamental precursor relationships, described the renal lesion seen in the patients, demonstrated that MMA is likely produced *de novo* or concentrated in the nervous system, showed that the disorder could be treated with precursor restriction and possibly hyperalimentation, localized the biochemical block to the methylmalonyl-CoA mutase step (MCM, also called MUT) and showed that the condition was co-factor responsive in some patients.

Over the past four decades, great progress has been made in understanding and treating this disorder. However, the challenges faced by physicians caring for the early patients, such as the propensity toward metabolic decompensation, growth and feeding problems, renal disease and premature death, still exist (*6–9*), as current treatment protocol outcomes continue to demonstrate substantial morbidity and mortality in the patient population (*10*). The larger questions of prognosis, the etiology of the complications, and the development and testing of improved therapies stand as a challenge for translational research endeavors. Along these lines, the use of model systems to study methylmalonic acidemia should help guide the development and testing of newer therapies for these disorders.

Impaired intracellular metabolism of vitamin B12 produces another group of disorders that feature methylmalonic acidemia, as well as (hyper)homocysteinemia. These conditions are named after the corresponding cellular complementation class — cobalamin C (cblC), D (cblD) and F (cblF) — and are also heterogenous, clinically and biochemically. The spectrum of clinical phenotypes associated with these conditions is incompletely understood and also under investigation in our clinical center. Figure 1 depicts an overview of the steps of intracellular cobalamin metabolism.

Phenotype and natural history of methylmalonic acidemia and related disorders

Since June 2004, we have evaluated 46 patients with methylmalonic acidemia and cobalamin syndromes to further define these phenotypes as part of a clinical research protocol (National Human Genome Research Institute Study 04-HG-0127 "Clinical and Basic Investigations of Methylmalonic Acidemia and Related Disorders, http://clinicalstudies.info.nih.gov/detail/A_2004-HG-0127.html"). We have been very fortunate to have patient and referring physician participation and are appreciative of the efforts every family has made to come here for a comprehensive evaluation. Our study group is one of the largest patient cohorts in the world with these disorders.

One major focus has been to analyze the biochemical parameters seen after solid organ transplantation, and correlate these findings with cellular, biochemical and molecular information to aid with decision making surrounding kidney, liver and combined liver-kidney transplantation procedures in patients with isolated methylmalonic acidemia/aciduria. To define the neurological syndromes present in the patients who have sustained metabolic strokes, we have examined anatomic and spectroscopic findings using a high field strength magnet, and correlated these results with neurological signs and clinical phenotypes. We have observed Wallerian degeneration, suggesting slow neuronal loss, downstream of the basal ganglia damage seen in some of the affected patients. The mechanism of this process and the tracts involved are unknown but these

observations provide new insights into clinical/imaging correlations and pathways to explore in model organisms.

Other ongoing clinical research endeavors include the definition of the natural history of the retinal lesions of cobalamin C (cblC) patients and the larger examination of genotype-phenotype correlations in all patient groups. Future efforts will focus on continued patient characterization, particularly transplant recipients, with the ultimate goal of assembling a large, representative cohort for prospective studies and the testing of newer therapies. An NIH conference on the treatment of isolated methylmalonic acidemia/aciduria is planned and will provide a forum to review current diagnostic evaluations and treatment protocols and to develop consensus guidelines for the future. Understanding the subtleties of the clinical phenotypes seen in the patients is important, because it provides direction for laboratory research and instructs the examination of existing models for findings related to patient care.

Model organism studies of methylmalonic acidemia and related disorders

Methylmalonic acidemia has been difficult to study in model organisms. Earlier efforts have used nutritional (*11*) and cobalamin-analog treatments (*12*) to induce methylmalonic aciduria in rats. These treatments are artificial and can produce an incomplete block, especially in the case of nutritional deficiency, causing combined methionine synthase and methylmalonyl-CoA mutase functional impairment. Establishing tractable model organism systems to study methylmalonic acidemia will be required to better understand the symptoms and complications associated with this group of disorders, develop new therapies and further define genes that may play a role in methylmalonyl-CoA and cobalamin metabolism. To date, knock-out murine models of methylmalonyl-CoA mutase have displayed a severe neonatal phenotype with uniform mortality by 24–36 hours of life (*1,13*). Organisms more amenable to genetic manipulations such as yeast and *Drosophila* do not possess cobalamin-dependent metabolic enzymes or utilize alternative pathways for propionyl-CoA metabolism, such as the methylcitrate cycle (*14*).

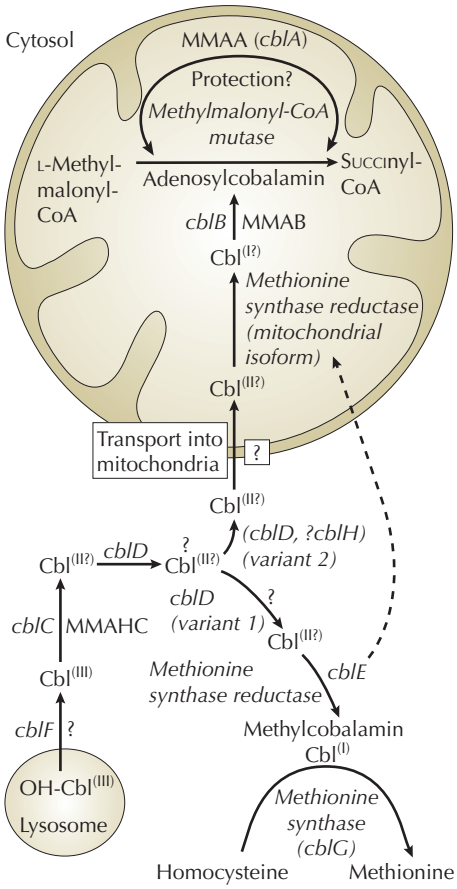

FIGURE 1. Pathway of cellular processing of cobalamin (CN-cbl, OH-cbl). The class and genes associated with isolated methylmalonic acidemia are *cblA* (MMAA), *cblB* (MMAB), *cblD variant 2 (unknown gene)*, *cblH* (*unknown gene*) and *mut* (MCM). Question marks indicate unknown genes or poorly defined cellular processes. The position of the MMAA gene product reflects the probable role that this gene has in protection of the mutase (*19*). A mitochondrial isoform of the methionine synthase reductase gene is thought to protect or interact with the product of the MMAB gene (*20*).

TABLE 1. Putative *C. elegans* homologues of genes involved in propionyl-CoA metabolism

Human protein	*C. elegans* protein gene	Protein % similarity/ e-value	Domains present in human protein	Domain(s) % similarity/ e-value
Propionyl-CoA carboxylase alpha subunit (PCCA) NP000273.1	WP:CE04451 F27D9.5 *pcca-1*	94.6/5.2e-215	Carbamoyl-phosphate synthase Lchain, ATP binding domain, pfam02786	89.8/2.00e-26
			Biotin caboxylase C-terminal domain pfam02785	100/1.00e-35
			Carbamoyl-phosphate synthase, N-terminal domain pfam00289	100/2.00e-27
			Biotin-requiring enzyme pfam00364	100/5.00e-12
Propionyl-CoA carboxylase beta subunit (PCCB) NP000523.1	WP:CE07269 F52E4.1 *pccb-1*	95.3/1.4e-212	Carboxyl transferase domain pfam01039	100/0.00e-00
Methylmalonyl-CoA Epimerase (MCE) NP115990.2	WP:CE09082 D2030.5 *mce-1*	79.6/2.3e-45	Glyoxalase pfam00903	100/7.00e-17
Methylmalonyl-CoA Mutase (MUT) NP000246.1	WP:CE30404 ZK1058.1 *mmcm-1*	97.8/7.29e-280	Methylmalonyl-CoA mutase B12 binding domain cd02071	100/5.00e-43
			Methylmalonyl-CoA mutase pfam01642	100/0.00e-00
Methylmalonic aciduria Type A (MMAA) NP758454.1	WP:CE31822 T02G5.13 *mmaa-1*	82.5/1.2e-89	ArgK protein pfam03308	100/3.00e-105
Methylmalonic aciduria Type B (MMAB-1) NP443077.1	WP:CE01159 C26E6.11 *mmab-1*	53.7/5.7e-35	Cobalamin adenosyltransferase pfam01923	97.6/4.00e-50
MMACHC	WP:CE34461 ZK546.17 *cblc-1*	52/5e-31	None proven	

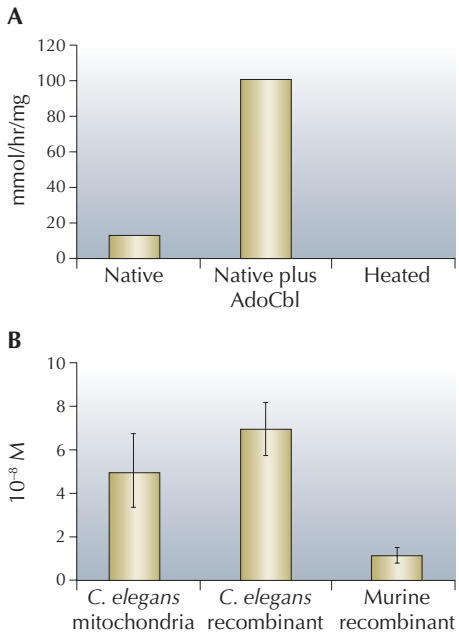

FIGURE 2. Properties of the methylmalonyl-CoA mutase enzyme from *C. elegans* (A) Activity and heat lability in purified mitochondrial preparations. (B) K_m for 5′ deoxyadenosylcobalamin of native and recombinant enzyme.

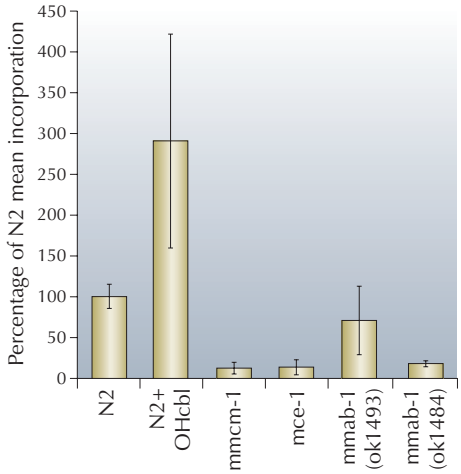

FIGURE 3. 1-[^{14}C]-propionate incorporation in *C. elegans* deletion mutants. 1-[^{14}C]-propionate incorporation of methylmalonyl-CoA mutase *mmcm-1(ok1637),* methylmalonyl-CoA epimerase *mce-1(ok243),* and *mmab-1* mutants *mmab-1(ok1493)* and *mmab-1(ok1484)* expressed as a percentage of the mean propionate incorporation of wild-type (WT = 6.8 nm propionate/mg protein/24hrs). Values are averages from 6 (N2, *mcmm-1*), 5 (*mce-1*) and 5 (*mmab-1*) independent experiments with triplicate or quadruplicate measurements of each sample. Error bars represent the mean ±SD. P-values vs. wild-type: *mmcm-1(ok1637)* = 2.9 E-22; *mce-1(ok243)* = 2.6 E-19, *mmab-1(ok1493)* = 0.008, *mmab-1(ok1484)* = 1.4 E-08, N2 plus OHcbl = 2.03 E-06.

Recently, we employed a combination of informatic, genomic, biochemical and metabolic analyses to identify and characterize genes that participate in methylmalonyl-CoA metabolism in the simple worm *Caenorhabdhitis elegans* (*15*). In addition to providing direct biochemical evidence for methylmalonyl-CoA mutase activity and adenosylcobalamin synthetic capacity, we utilized genomic approaches to demonstrate that *C. elegans* can be used to define the function of gene products previously suspected to participate in methylmalonyl-CoA metabolism in man, particularly methylmalonyl-CoA epimerase (MCEE).

Informatics was used to identify putative homologues (TABLE 1) that were further studied using RNAi and metabolite analysis. Biochemical studies on recombinant *C. elegans* MCM and native mitochondrial preparations from the animals provided evidence for a functional mutase reaction (FIG. 2) and further demonstrated that adenosylcobalamin synthetic capacity from hydroxycobalamin was part of the mechanism by which these animals utilized cobalamin. Deletion mutants at the major steps were isolated and characterized and convincingly demonstrated that loss of the MCEE enzyme caused a severe impairment in C^{14}-propionate incorporation (FIG. 3). This result, obtained using only model organism genomics and biochemistry, has provided convincing evidence that MCEE is important in the intermediary metabolism of propionyl-CoA and methylmalonyl-CoA. Indeed, two recent reports have described patients with methylmalonic acidemia who have MCEE mutations that are suspected to inactivate the enzyme (*16,17*). Our *C. elegans* experiments have carefully characterized of a number of mutant strains that other investigators might use for future studies on methylmalonic acidemia.

Murine models of methylmalonic acidemia

We have generated a targeted deletion of methylmalonyl-CoA mutase (*Mut*) to produce a mouse model of vitamin B12 non-responsive methylmalonic acidemia that displays neonatal lethality, similar to that observed in another murine model (*1,13*). Using the mice, we have examined the tissue sources of MMA production and used this information to understand the clinical observations of persistent of methylmalonic acidemia in patients who undergo solid organ transplantation. These insights have refined our understanding of the post-transplant physiology in methylmalonic acidemia and suggested new pathways for modulation of metabolism to lower circulating MMA levels.

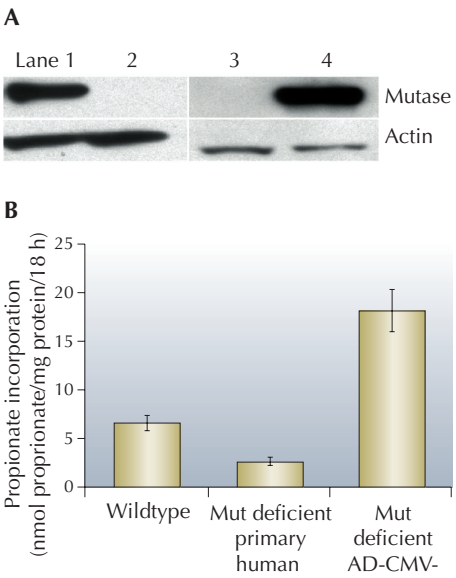

FIGURE 4. Expression and functional studies of methylmalonyl-CoA mutase in primary *mut^o* human hepatocytes. A. Western analysis of protein lysates from primary human hepatocytes probed with methylmalonyl-CoA mutase antibody (78 kDa, labeled mutase) and cross-reactive β-actin loading control (47 kDa, labeled actin). Lane 1: human control whole liver extract (20 µg) Lane 2: *mut^o* whole liver extract (20 µg); Lane 3: human primary *mut^o* primary human hepatocytes (10 µg); Lane 4: adenoviral corrected *mut^o* primary human hepatocytes (10 µg). B. Human hepatocytes (wild type, *mut^o* and *mut^o* corrected with adenovirus) were assayed for 1-[^{14}C]-propionate incorporation over 18 hours after viral incubation for 48 hrs. Activity was normalized to total protein content of the extracts. The samples were analyzed in triplicate. The symbols represent the mean ±SD.

The creation of murine models to replicate partial deficiency methylmalonic acidemia has been initiated using human patient studies to guide mutation selection. An unusual change in the *MUT* gene was identified in an affected patient enrolled in our clinical protocol. The patient did not respond to high-dose cobalamin therapy *in vivo* but did exhibit a mild increase in cellular enzymatic activity when the patient cells were tested *in vitro*. We determined that the mutant enzyme harbored by this patient exhibits a K_m for adenosylcobalamin 10-fold greater than that of the wild-type enzyme, but has a normal V_{max}. The homologous mutation has been introduced into the murine gene and used to create a partial deficiency model of methylmalonic acidemia. The resulting animals have methylmalonic acidemia/aciduria but grow and develop normally and are under study as an inducible model of the metabolic changes seen in the more severely affected Mut$^{-/-}$ animals.

Gene and cell therapy in murine models of methylmalonic acidemia

We have produced and validated a variety of viral vectors to deliver the *Mut* gene in cell culture experiments. To demonstrate the efficacy of viral correction, we have successfully corrected primary human methylmalonyl-CoA mutase deficient hepatocytes (*18*). The extent of correction in the affected human hepatocytes was complete (Fig. 4A, lane 4) and produced a 3-fold increase in propionate metabolism over control hepatocytes after viral correction (Fig. 4B). These studies firmly demonstrate proof of principle for hepatocyte-directed gene therapy strategies in methylmalonic acidemia. Gene delivery and cell therapy experiments will be the subject of future studies.

Conclusions

Integrated, cross disciplinary approaches have led to the development of model organism systems that will be useful to study methylmalonic acidemia using experimental strategies. Patient studies will continue to inform and direct laboratory research.

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Propionic acidemia: what we know and don't know about the pathophysiology

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In spite of the identification of many genes responsible for inborn errors of metabolism, their protein products and the relevant biochemical pathways, we still have a poor understanding of how a primary defect in a single biochemical function causes clinical disease. In propionic acidemia, the accumulation of propionyl-CoA within mitochondria is probably responsible for most, if not all of the clinical signs and symptoms of the disease. Yet, the path from this biochemical abnormality to symptoms such as muscle weakness, developmental delay, anorexia, cardiomyopathy and bouts of ketoacidosis is elusive. Here I summarize what is known and unknown about the pathophysiology of propionic acidemia and highlight areas where research in needed in order to better understand this severe disorder and develop better treatments to improve the quality of life and longevity of patients.

Amino acids that derive from dietary or endogenous proteins can either be used for synthesis of new proteins or be degraded and oxidized to ammonia carbon dioxide and water while producing energy. Even if an individual consumes very little protein, some of the amino acids from the diet and those generated in the organism by cellular metabolism are destined to degradation. In order for amino acids to be completely degraded, a cascade of several enzymatic reactions needs to

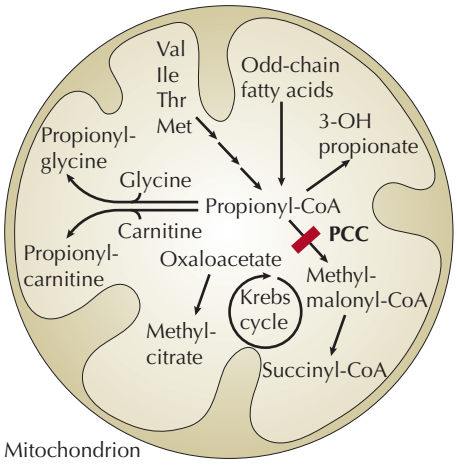

FIGURE 1. Biochemical pathophysiology in propionic acidemia.

work in sequence, where the product of one enzyme is used as a substrate for the subsequent enzyme in the pathway.

The amino acids relevant to propionic acidemia are valine, isoleucine, methionine and threonine. These four amino acids, along with fatty acids with an odd number of carbon atoms and a component of the cholesterol molecule are all sequentially degraded until propionic acid linked to coenzyme A is produced. Thus, propionyl-CoA (PCoA) is a common degradation product from the oxidation of all these molecules, which occurs in liver, muscle, kidney, brain and many other organs (FIG. 1).

PCoA is formed inside the mitochondria, the energy generators of cells, where the Krebs cycle and other important enzymes and functions reside. However, this oxidation product is normally present in very low concentrations because it is immediately converted to methylmalonyl-CoA by a biontinylated enzyme, PCoA carboxylase (PCC). Methylmalonyl-CoA is readily converted to succinyl-CoA by another enzyme — methylmalonyl-CoA mutase. Succinyl-CoA is then oxidized by the Krebs cycle or converted to glucose.

Biochemical observations in propionic acidemia

Patients with propionic acidemia (PA) have mutations in one of the two genes that code for the PCC protein (Ugarte *et al.*, 1999). As a result, the PCC enzyme is either absent, reduced or malfunctions. Thus, propionic acidemia is caused by a defect in a single mitochondrial enzyme reaction that prevents the entry of PCoA into the degradation cascade, altering the biochemical equilibrium (homeostasis) within the mitochondria and the cell.

PCoA is a large and highly charged molecule that is unable to readily leave the mitochondria; for this to happen, the CoA molecule has to be removed first, either by enzymatically breaking it off from the propionyl moiety, or by replacing the CoA with carnitine to form propionylcarnitine, which can readily cross the mitochondrial membrane, leave the cell into the bloodstream, and subsequently be excreted in the urine. One can therefore easily surmise that, when the PCC reaction is blocked, PCoA will accumulate within the mitochondria and this accumulation is likely to be the culprit responsible for the pathophysiology of PA.

By contrast to propionate, the levels of which may be 100-fold higher in the blood of PA patients than in healthy people (Hommes *et al.*, 1968), PCoA is not found in blood or the urine of patients. In addition to the fact that PCoA cannot cross the mitochondrial membrane, CoA derivatives are quite unstable and degrade under physiological conditions. There is, however, indirect evidence for the accumulation of PCoA within the mitochondria.

Urine organic acid analysis in patients with PA reveals the presence of large amounts of methylcitrate (Ando *et al.*, 1972), a compound that is considered synonymous with the diagnosis of PA. Under normal conditions, little methylcitrate is produced, and it is not known what the metabolic role of this compound (if any) might be. In PA, methylcitrate seems to be the result of PCoA condensation with oxaloacetate, a Krebs cycle intermediate (Ando *et al.*, 1972a). This observation tells us that, when PCoA concentration is markedly increased, this molecule can act as substrate of enzymes that normally do not interact with it. Thus, in this case, the Krebs cycle enzyme citrate synthase, which normally uses acetyl-CoA and oxaloacetate to produce citrate, is now flooded with high concentrations of PCoA, which then outcompete acetyl-CoA as substrate, producing methylcitrate instead.

Other biochemical markers of PA are likely a result of similar mechanisms. 3-hydroxypropionic acid, which is excreted in relatively large amounts in the urine of patients with PA, seems to derive from beta-oxidation, a normal oxidation pathway of free fatty acids (Ando *et al.*, 1972b). Thus, the high concentrations of PCoA allow it to inappropriately enter the mitochondrial beta-oxidation pathway generally reserved for fatty acids with an even number of carbons. Propionylglycine is probably the product of the enzyme glycine *N*-acylase, which normally produces hippuric acid from benzoyl-CoA and glycine, and now uses PCoA instead (Rasmussen *et al.*, 1972a,b). Similarly, propionylcarnitine is produced in lieu of acetylcarnitine (the most abundant acylcarnitine species) due to the high concentration of PCoA, which displaces some of the acetyl-CoA molecules from the enzyme carnitine acetyltranferase (Roe *et al.*, 1984).

It is also possible to speculate that, because many CoA molecules are "trapped" as PCoA, a relative CoA deficiency could develop within the mitochondria, impinging on other processes that depend on CoA. Similarly, as large amounts of endogenously produced free carnitine are bound with propionic acid (propionylcarnitine) that is lost in the urine of PA patients, a state of free-carnitine deficiency could develop (DiDonato *et al.*, 1984), leading to the dysfunction of systems that use carnitine (e.g. fatty acid metabolism). Indeed, the livers of patients with PA contain excessive fat, a likely consequence of possible interference of the disease with normal fatty acid oxidation. In addition, there is presence of abnormal long-chain fatty acids with an odd number of carbons that might be derived from enzymatic reactions involving PCoA instead of acetyl-CoA (Hommes *et al.*, 1968).

Other biochemical observations in PA are less readily explicable, but may still be all caused by the accumulation of PCoA. Ketosis and metabolic acidosis can be caused by interference with normal ketone body utilization. Elevated plasma glycine levels seen almost universally in PA patients could result from inhibition of one or more proteins in the glycine degradation pathway (Hillman and Otto, 1974). Hyperammonemia, which is seen frequently during metabolic decompensation, is possibly the result of a secondary effect on a urea cycle component, likely *N*-acetylglutamate (Coude *et al.*, 1979).

Patients with PA show other biochemical aberrations, including a number abnormally elevated organic acids such as Krebs cycle derivatives or intermediates in the degradation of leucine. These may result from a general mitochondrial dysfunction, indicating that the accumulation of PCoA causes severe disturbances in many metabolic processes.

Notwithstanding the above considerations, there is evidence that the toxic compounds that are generated in PA can be detoxified through cleansing

of the blood. In the womb, babies with PA appear to develop well and, when they are born, they are indistinguishable from normal newborns. This would seem to indicate that the maternal circulation and metabolism are able to detoxify PA-related toxins. Thus, it is likely that propionic acid and other metabolites that are generated in PA can pass into the bloodstream, provided that the blood is being continuously cleansed to maintain the gradient between blood and tissues. The same can also be accomplished effectively, yet temporarily, by hemodialysis treatment of severe acute decompensations.

From biochemistry to clinical manifestation

The major challenge in medicine is to link biochemical abnormalities seen in various diseases to the clinical manifestation. If we could connect the biochemistry of disease to clinical signs and symptoms, we should be able to develop better therapies to improve the quality of life and survival of patients. Linking genes and proteins on one end with the clinical phenotype on the other has been, and remains the weakest link of medical science. The reason this is so has to do with the complexity of the biological systems and their incalculable components and factors. It is mind-boggling that we were able to find the cause for PA almost 40 years ago, but we still have only a rudimentary understanding of how a single enzyme deficiency can wreck havoc on so many organ systems in the patients.

We do not know whether the biochemical aberrations that are observed in PA exert their effect mainly locally, causing dysfunction of the organ in which they are generated and/or whether toxicity exerted on some organs arrives via the bloodstream. For example, does the toxicity of PA on the brain results from the generation of toxic metabolites within the brain itself, or is it caused by toxic metabolites that are generated in other organs (liver, muscle, kidney, intestine). Indeed, intestinal bacteria produce propionic acid from their metabolism, some of which is absorbed into the blood (Thompson *et al.*, 1990a). This may be a significant source of toxicity for other organs in patients with PA, as temporarily reducing the number of intestinal bacteria with antibiotics has been reported to ameliorate the metabolic crises (Thompson *et al.*, 1990b).

Some insight into the answer to the question of whether the generation of toxic compounds that leads to organ damage occurs locally or distally could be obtained from genetically engineered mouse models of PA, which currently exist (Miyazaki *et al.*, 2001), and from experience of patients with PA who underwent liver transplantation, in whom an affected organ that generates significant amounts of propionic acid has been replaced by a normal liver (Barshes *et al.*, 2006).

Let us consider the main features of the clinical phenotype of PA and explore if and how they can be linked to biochemical mechanism, keeping in mind that all of them result from a single blocked biochemical reaction.

PA manifestations can be arbitrarily divided into acute and chronic. Although some of the acute manifestations could just reflect more extreme aberrations of the same abnormalities that are present in the chronic phase of the disorder, one needs to explain the rapidity and insidiousness of acute attacks. These are sometimes associated with concurrent, and not severe, viral or bacterial infections, but many times no apparent trigger can be identified. It is possible that there exists a pathological threshold that, when crossed, sets in motion a vicious cycle that leads to rapid metabolic decompensation. In an unpublished observation, I have observed that days before acute attacks, the ratio between propionylcarnitine and free carnitine in the blood increases from its already abnormal baseline. When I tried to increase the carnitine supplement dose, it did not seem to make a difference and the patient would still develop an acute ketoacidosis attack within a few days requiring hospitalization and intravenous treatment. Does this tell us that there is a slow phase of decompensation followed by a rapid one? Only well controlled research studies in a sufficiently large number of patients could confirm this observation, allowing us to develop better prevention strategies.

When acute exacerbation occurs, it is usually associated with ketoacidosis, but hyperammonemia does not seem to be a required component of an acute attack. The exact path from the accumulation of PCoA to acidosis and ketosis, in spite of being intuitive, is not well understood. The acidosis is associated with an increased anion gap, thus pointing to increased blood levels of circulating acids, such as ketone bodies (acetoacetate and 3-hydroxybutyrate) and others. It is possible, even likely, that PCoA interferes with ketone utilization (Dutra *et al.*, 1991) and, when PCoA reaches a certain threshold, ketones in the blood rise rapidly leading to significant acidosis.

More research is needed to understand the sequence of these events and the specific enzymes affected, and how to intervene most effectively. Currently, we use what I refer to as a "treatment dogma", as much of it does not rely on evidence-based medicine or well-controlled studies, but on "medical reasoning" and a trial and error approach. For example, we know that, following treatment with intravenous fluids, large amounts of glucose and carnitine, the acute attacks subside within a few days. Frequently, dialysis is used to treat extreme decompensations, usually in the newborn period. But we have little knowledge of what needs to occur at a mechanistic level for the attack to subside and whether all therapeutic measures that we take are actually useful. Short-term administration of anabolic hormones such as growth hormone (Marsden *et al.* 1994) or androgens might be useful but, again, their effect needs to be studied in a systematic way.

As acute exacerbations are frequently associated with abnormal signs of the central nervous system, from various degrees of lethargy to full coma, one can assume that each attack causes incremental brain damage. If these attacks could be prevented or minimized, a better long-term outcome should follow. Patients with PA seem to have abnormalities in basal ganglia and the brain's white matter (Brismar and Ozand, 1994). As the basal ganglia and the brain stem have the highest oxygen consumption in the brain and are affected in disorders of energy metabolism, it is not unreasonable to assume that that energy production is compromised in the brain cells of patients with PA (Chemelli *et al.*, 2000).

Similarly, the hypotonia, muscle weakness and myopathic features observed in patients with PA point to an energy consumption-related mechanism. However, exactly how energy deficits develop in PA and why significant lactic acidosis is not part of the picture while it is commonly observed in disorders of energy metabolism remains unclear. Patients with severe PA tend to develop a hypertrophic cardiomyopathy later in life (Massoud and Leonard, 1993). Although one is tempted to ascribe this sign to mitochondrial dysfunction and perhaps to carnitine deficiency, the reason for its late development in the course of the disease is unclear and requires detailed study.

Every health professional who has managed patients with PA has likely observed the chronic, severe anorexia

of these patients. While anorexia is not uncommon in metabolic conditions, the disinterest in food manifested by patients with PA is striking, universally requiring enteral feeding. The dependence of the patients' health on maintaining adequate caloric intake only exacerbates this problem. Yet, we have little understanding of the pathophysiology. Is the anorexia the result of a neurodevelopmental problem? In other words, are brain connections that need to develop early after birth compromised by the abnormal brain biochemical milieu? Or is anorexia the result of never allowing the patients to develop a sense of hunger (and therefore to regulate their own food intake), as their lives from early on depend on constant caloric intake that is often closely monitored? Although some clues to abnormal neurotransmitter physiology have been put forward, this clinical problem remains one of the most frustrating to patients, their families and health professionals. We could not rule out that some of the poorly explained clinical signs we observe in patients with PA are side effects of our management. As we have to resort to treatment based on "medical reasoning", our common sense might prove incorrect because we do not have an in-depth understanding of the paths that lead from the biochemical abnormality to the clinical manifestation.

Liver transplantation has been tried in a dozen patients with propionic acidemia as a measure to provide normal propionate metabolism (Barshas *et al.*, 2006). As the biochemical defect is present in other tissues, one would not expect complete reversal of the metabolic disorder, as is seen in other genetic conditions that affect the liver almost exclusively. In spite of this, liver transplantation has improved the outcome of patients, reduced the number of acute episodes and improved the metabolic abnormalities. These observations support the role of the liver as having a major role in the pathophysiology of PA and should be a target of future new treatments.

It is known that the circulating white blood cells, especially neutrophils, in patients with PA are frequently reduced in number. It seems that propionic acid in high concentrations can inhibit the growth and maturation of bone marrow cells (Stork *et al.*, 1986). As one of the main roles of white blood cells is to combat bacteria, patients with PA are susceptible to infections. This specifically increases the risk of infections from central venous catheters that need to be frequently used in patients, as vascular access is so difficult in many of them. Again, the mechanism by which bone marrow suppression occurs in PA is unknown.

Concluding remarks

We have important information about the genetic, biochemical and clinical aspects of PA, including the genes and proteins involved, how to diagnose the disorder and how to gauge its clinical severity. The survival of patients with PA has been increasing thanks to more preventive and aggressive treatments, and to the development of specific medical foods. However, we still have a long way to go to prevent the mental retardation and other disabilities that are almost universal in PA. We have a large gap in knowledge about the path from biochemical abnormality to clinical signs and symptoms, both at a cellular level, the organ level and the whole patient. As effective gene therapy for multiple organs, the ultimate tool for curing PA, remains elusive, we need to focus our efforts on improving our understanding of the factors involved in the pathophysiology and the way they interact. This can only be accomplished by innovative, meticulous research. It would be highly desirable to create a research network for PA, methylmalonic acidemia and other similar organic acid disorders to be able to study larger numbers of patients on identical protocols, as has recently been started for other rare diseases with federal funding.

Acknowledgements

This article is dedicated to the patients with PA and their parents and families from whom I have learned to listen carefully, not to give up, to challenge the dogma, to appreciate my limited understanding of the complexity of the disease, and to realize that the treatments that we currently can offer are grossly insufficient and that we need to do better.

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Therapeutic approaches to propionic acidemia

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In vitro evidence indicates that the activity of mutant propionyl CoA carboxylase — the enzyme affected in people with propionic acidemia — increases in the presence of protein or chemical chaperones. Propionic acidemia may therefore be a 'conformational disorder', opening the door to new therapeutic opportunities.

Four different biotin-dependent carboxylases play a central role in mammalian metabolic pathways, such as oxidation of odd-chain fatty acids, catabolism of branched amino acids, fatty acid synthesis and gluconeogenesis. One of these is propionyl CoA carboxylase (PCC), which catalyzes the conversion of propionyl CoA to D-methylmalonyl CoA in the mitochondrial matrix (Fenton *et al.*, 2001).

This enzyme consists of two nonidentical subunits — α and β — encoded by two different nuclear genes, designated *PCCA* and *PCCB*, respectively. Structural studies of the human enzyme have indicated that α PCC is 72 kDa in size while β PCC is 54 kDa. Overall, PCC has an $\alpha_6\beta_6$ structure (Fenton *et al.*, 2001; Haase *et al.*, 1984). Mutations in either gene result in an autosomal recessive disease, propionic acidemia (PA), which usually presents as a life-threatening ketoacidosis in the neonatal period with protein intolerance, vomiting, failure to thrive, lethargy, and profound metabolic acidosis symptoms. This disease can result in mental retardation and can be sufficiently severe to cause neonatal death (Fenton *et al.*, 2001).

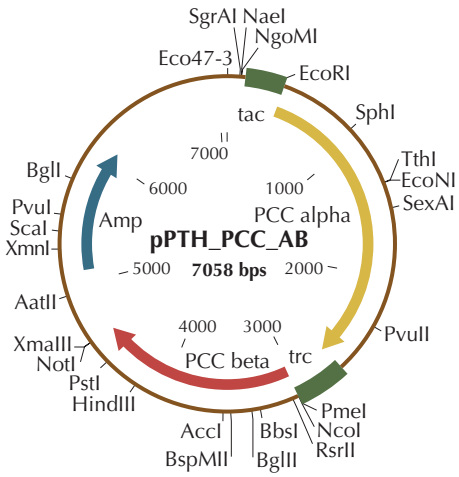

FIGURE 1. Human PCC expression construct pPTHPCCAB.

The cDNAs for α and β subunits have been cloned and the structure of the genes has been elucidated (Campeau *et al.*, 2001; Lamhonwah *et al.*, 1994; Ohura *et al.*, 1993; Rodriguez-Pombo *et al.*, 1998; Stankovics and Ledley, 1993). With the sequence information available, it has been possible to identify mutations from PA patients (Lamhonwah *et al.*, 1990; Tahara *et al.*, 1993; Ugarte *et al.*, 1999), and many new mutations have been found recently in different ethnic groups (Kim *et al.*, 2002; Perez *et al.*, 2003). Currently, approximately 52 and 53 mutations have been reported in the PCCA and PCCB genes, respectively (for a continuously updated list of all reported PCC mutations see our website at http://www.uchsc.edu/cbs/pcc/pccmain.htm).

Expression of recombinant human PCC in *E. coli*

We have developed a human PCC expression construct designated pPTHPCCAB (FIG. 1). This plasmid has been designed to facilitate the simultaneous and balanced expression of both PCC α and β subunits in *E. coli*. Subsequent experiments in our laboratory have shown that successful assembly of wild-type PCC in *E. coli* is greatly aided by the presence of the molecular chaperonins GroES and GroEL (Kelson *et al.*, 1996a). There are 54 mutations currently identified in the beta subunit of human PCC. To date, we have engineered a total of 21 of these mutations (primarily missense) into our PCC expression construct and studied their expression products in *E.coli*. The initial analyses of the mutant recombinant enzymes have been all done in semi-purified extracts. Nevertheless, we were able to conclude from the data obtained on all 21 mutants that at least half of them should be amenable to purification and further biochemical and physical characterization at the level of pure proteins.

Currently, we have purified a total of 11 mutants and one polymorphic variant form of PCC (FIG. 2). About one third of these were proteins with significant residual PCC activities (R165W, E168K, P228L, R410W and the polymorphism A497V), while the remainder were inactive (R44P, S106R, G131R, G198D, V205D, T428I, and M442T) (TABLE 1). The T428I and the R410W are the most prevalent PCC mutations in East Asia (Korea and Japan, in particular). We have further characterized three of the purified PCCs containing the pathogenic mutations (R410W, R165W, and E168K) and one PCCB polymorphism (A497V) in order to elucidate the potential structural and functional effects of these substitutions. The purified R165W and E168K had as much as 42 and 32% of wild-type activity, respectively, while the polymorphic variant, A497V had 138% of wild-type activity. No significant differences have been observed in K_m values for propionyl CoA, oligomeric assembly and secondary structure among these PCCs using PCC assays, size-exclusion chromatography and far ultraviolet circular dichroism. However, the variant PCCs were less thermostable than the wild type. Considering the profound effect of the co-expressed chaperone proteins on PCC folding, assembly, and activity, we believe that the pathogenic nature of these mutations

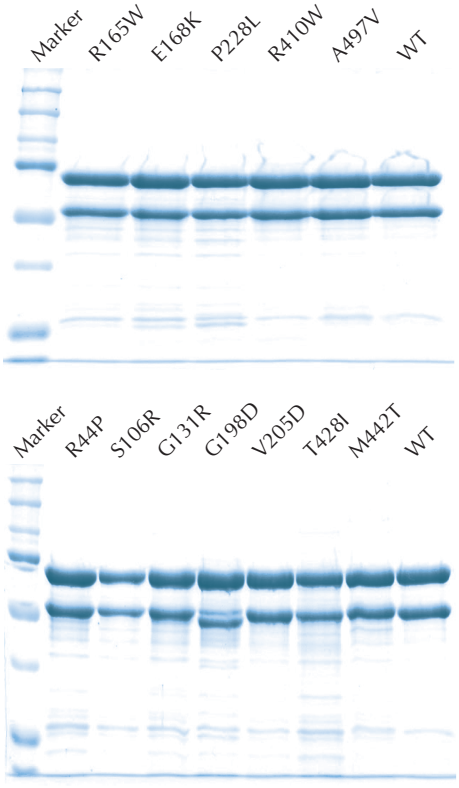

FIGURE 2. SDS PAGE of the purified PCC enzymes. The active enzymes are shown above while the inactive ones are at the bottom.

TABLE 1. Activities of purified mutant PCCs

Mutations	SA (pmol/min/mg protein) × 10^{-6} Mean ±SE	% of WT
WT R44P, S106R, G131R, G198D, V205D, T428I	23.35 ±1.11	100.00
M442T	0.00	0.00
R165W	9.93 ±0.37	42.50
E168K	7.54 ±0.09	32.30
R410W	9.43 ±0.06	40.41
P228L	0.59 ±0.10	2.55

is more likely due to lack of assembly rather than any disruption of the catalytic process (Jiang *et al.*, 2005). Taken together with our preliminary results on chemical chaperones (see below), these findings indicate that some PCC mutations exert their pathogenic effect due to an inability to assemble correctly in patient's cells and that functional replacement of our GroES/EL chaperone system with chemical chaperones has highly significant potential to restore activity to these mutant forms of PCC in subjects with PA (Chloupkova *et al.*, 2002).

Is PA a conformational disorder?

Protein folding is an essential cellular process, which can be impaired by mutations, errors in translation or many environmental factors such as prions,

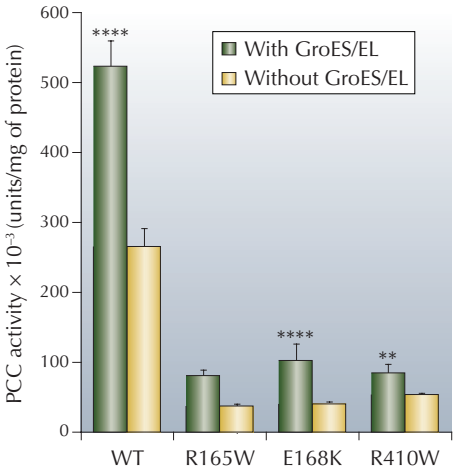

FIGURE 3. Expression of PCC with and without GroESL. The values represent the mean and SE derived from 3–19 independent experiments. The significant levels of the differences between experiments with and without GroES/EL are shown as ** and **** for P< 0.01 and P<0.0001, respectively.

oxidative, osmotic or thermal stress (Soto, 2001). Misfolded proteins are prone to increased ubiquitinylation and rapid degradation (Kisselev and Goldberg, 2001), and/or to aggregation with subsequent formation of aggresomes (Johnston *et al.*, 1998). Diseases arising by such a mechanism are collectively named conformational diseases (Carrell and Lomas, 1997; Soto, 2001).

Protein chaperones direct the folding of polypeptides into functional proteins, facilitate developmental signaling and, as heat-shock proteins (HSPs), can be indispensable for survival in unpredictable environments. Chaperones do not alter genotype, but rather the expression of genetic variation as phenotypic variation. Protein folding, maintenance and repair are highly specialized cellular functions (Bukau and Horwich, 1998; Frydman, 2001; Hartl and Hayer-Hartl, 2002). Chaperones act by preventing the formation of promiscuous, but energetically stable, associations in, or between, non-native polypeptides; many use ATP-driven cycles of binding and release to destabilize non-native intermediates. This gives the polypeptides repeated opportunities to reach a stable mature fold (Rutherford, 2003).

Several recent studies have described the use of a group of low-molecular-weight compounds to reverse the mislocalization and/or aggregation of proteins associated with human disease. These compounds, which include polyols such as glycerol, trimethylamines such as trimethylamine-*N*-oxide (TMAO) and amino acid derivatives, have been called "chemical chaperones". Recent studies have suggested that other compounds, such as 4-phenylbutyric acid (PBA) and membrane-permeable forms of enzyme antagonists, ligands or even substrates, can also act as chemical chaperones for misfolded or mislocalized enzymes. The mechanisms by which chemical chaperones function are not fully understood but are thought to include stabilization of improperly folded proteins, reduction of aggregation, prevention of nonproductive interactions with other resident proteins and alteration of the activity of endogenous chaperones. Chemical chaperones of the glycerol, TMAO and PBA class have general effects on multiple proteins while antagonists, ligands and substrates are thought to affect only the specific proteins with which they interact (Perlmutter, 2002).

With this in mind, ever since we started to express PCC in *E. coli* (Kelson *et al.*, 1996b), we have used a co-expressed

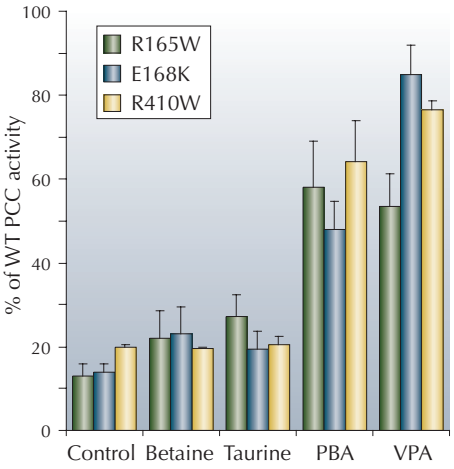

FIGURE 4. The impact of four chemical chaperones on three mutant PCCs. Control signifies no added chaperone. The values represent percentages of WT activity in the absence of GroES/EL and are shown as a mean ± SE derived from 3–8 independent experiments. The significant levels of differences between no treatment and PBA or VPA treatments yielded P<0.0001.

GroESL to aid in its assembly. FIGURE 3 illustrates the response of PCC to GroESL. The activity of the wt and the mutants approximately doubles in the presence of GroESL. The activity of this "chaperoned" PCC persists so that PCC mutants can be purified from crude *E. coli* extracts to homogeneity and the active enzymes do retain very high residual activities (TABLE 1 and FIG. 2). For all further preliminary studies with chemical chaperones we have concentrated on three βPCC mutants, R165W, E168K, and R410W, that were clearly impaired in folding/assembly rather than in the catalytic steps (Jiang *et al.*, 2005).

Looking for chemical chaperones for PCC

An initial screen of several candidate chemicals was performed using wild-type recombinant human PCC expressed in *E. coli* without GroESL but with the individual chemicals in the growth medium. As can be seen in TABLE 2, the presence of 5% glycerol in the medium had clearly a negative impact on PCC activity in cell extracts. While DMSO and TMAO had no influence on PCC activity, betaine and taurine had a clearly favourable influence. By far, the most pronounced effects were seen with 4-phenylbutyrate (PBA) and valproic acid (VPA). The ~2.5-fold increases in activity roughly paralleled those observed with GroESL. We then performed a screen of the three selected mutants with four of the most promising chemicals from the

TABLE 2. Impact of chemicals on wild-type PCC activity

Chemicals	PCC activity	
	Specific activity (pmol/min/mg protein) $\times 10^{-3}$	Relative activity (%)
None	296.6 ±44.0	100.0 ±14.8
Betaine	375.7 ±93.0	126.7 ±31.3
Taurine	422.1 ±122.0	142.3 ±41.2
Glycerol	170.5 ±42.2	57.5 ±14.3
DMSO	294.1 ±78.1	102.9 ±26.3
TMAO	305.4 ±78.0	99.2 ±26.4
PBA	701.5 ±71.9	236.5 ±24.3
VPA	750.3 ±124.2	252.9 ±41.9

preliminary wild-type PCC testing.

The response of the mutant PCCs was more pronounced that the one observed for the wild-type enzyme. FIGURE 4 shows that PBA and VPA yielded activities that approached 60 and 80% of wild-type activity, respectively.

Conclusion and outlook

We have found that some misfolded PCC mutants respond robustly to chaperones. The most potent response so far has been observed with PBA or VPA and in combinations of the molecular chaperone GroESL and the chemical chaperone VPA. The combined treatment of the R165W mutant increased the activity ~9-fold reaching >90% of the untreated wild-type enzyme activity. In addition to characterizing the enzyme in crude extracts, we have purified the wt and R165W enzymes following four different treatments. The results with the purified enzymes showed that the changes in the PCC dodecamer conformation are permanent (results not shown). The experiments with the purified enzymes have provided evidence that the chaperoned enzymes are expressed in increased amounts, and that they are catalytically more efficient and conformationally more stable to heat treatments.

Among the chemical chaperones we used in our preliminary trials, three of them are already in clinical use for different conditions. Betaine has been used extensively in pyridoxine nonresponsive homocystinuria as a donor of a methyl group for remethylation of homocysteine to methionine by betaine homocysteine methyltransferase. PBA (4-phenylbutyric acid) is already in routine use for ameliorating hyperammonemia in subjects with PA and urea cycle disorders. It is also in clinical trials for treatment of solid tumors, for cystic fibrosis and for spinal muscular atrophy. VPA is the treatment of choice for some forms of epilepsy. It has also been used for treatment of spinal muscular atrophy.

Future studies

We will continue to study any responses of recombinant mutant PCC enzymes to treatments with chaperones in *E. coli*. We will measure the amounts of *PCCA* and *PCCB* mRNAs in our skin fibroblast lines to determine which ones should be tested for a treatment with chemical chaperones. We have recently obtained Arimoclomol, a co-inducer of heat shock proteins that is being tested for the treatment of amyotrophic lateral sclerosis and has been shown to have cytoprotective effects including a murine model of ischemia and wound healing in the diabetic rat. We are in the process of testing this compound in our previously characterized constructs and in skin fibroblast cultures of propionic acidemia patients.

It is our hope that one or more of the compounds that we are currently testing will eventually lead to clinical trials in order to apply these promising treatments to patients suffering from these diseases.

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Gene therapy for propionic acidemia

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Propionic acidemia (PA) is a metabolic genetic disease that occurs in ~1 in 35,000 live births in the United States and up to 1 in 3,000 births in Saudi Arabia. This disease causes metabolic acidosis, ketosis, vomiting, lethargy, mental retardation and death. This metabolic decompensation occurs in response to the consumption of normal levels of proteins in the diet, but can also be precipitated by other stimuli. PA is caused by deficiency in either the α or β subunits of the mitochondrial enzyme propionyl CoA carboxylase (PCC), encoded by the PCCA and PCCB genes. Since there is currently no cure for PA, we discuss the feasibility and pilot testing of gene therapy to correct this disease in mice deficient for PCCA.

Propionyl CoA carboxylase deficiency

Propionic acidemia (PA) is an autosomal recessive inborn error of metabolism estimated to occur in ~1 in 35,000 live births in United States and perhaps up to 1 in 3,000 incidence in Saudi Arabia (reviewed in Ugarte *et al.*, 1999). The disease is heterogeneous in clinical manifestation, presenting within the first week of life or after 6 weeks. Clinical features include metabolic acidosis, ketosis, vomiting and lethargy. Mental retardation and median survival of 3 years is observed in early-onset patients, whereas late-onset patients may present with movement disorders and dystonias.

PA is caused by deficiency in the mitochondrial enzyme propionyl CoA carboxylase (PCC). PCC is involved in the metabolism of branched chain amino acids, odd-numbered chain length fatty acids, cholesterol and other metabolites (Fig. 1A). In the absence of PCC activity, metabolites build up in patient cells and in their circulation causing metabolic ketoacidosis (Fig. 1B). This ketoacidosis is complicated by hyperammonemia that is produced by the inhibition of *N*-acetylglutamate synthetase by excess propionyl-CoA (Coude *et al.*, 1979). The presence of byproducts in the blood or urine including propionyl-carnitine, 3-hydroxypropionate and methylcitrate are also indicators of failure of this enzyme (Fig. 1B). Because of its role in amino acid metabolism, consumption of normal protein levels in the diet can exacerbate the symptoms of PA.

PCC is a biotin-dependent enzyme consisting of six α and six β subunits (Lamhonwah *et al.*, 1986). The PCCA protein is covalently biotinylated on lysine 569 near its carboxy terminus by the biotin ligase holocarboxylase synthetase (Saunders *et al.*, 1982). Once conjugated to PCCA, biotin mediates the transfer of carbon dioxide from bicarbonate to propionic CoA (Fig. 1A and Chapman-Smith and Cronan, 1999). In cells deficient for the α subunit, the β subunit is unstable (Lam Hon Wah *et al.*, 1983). The α subunit is encoded by the PCCA gene on human chromosome 13 and the β subunit is encoded by the PCCB gene on human chromosome 3. Most mutations in the PCCA and PCCB in patients are thought to be point mutations or small insertions or deletions (Desviat *et al.*, 2004). However, splicing mutations have been more recently identified that skip whole exons (Desviat *et al.*, 2006), suggesting that larger regions of the proteins could be lacking in some PA patients.

There is currently no cure for PA. Patients are currently managed with intensive alkali therapy and protein restriction, as normal protein consumption can drive severe metabolic ketoacidosis. Administration of excess carnitine can attenuate symptoms, as carnitine can act as a carrier to remove propionyl-CoA from cells (Roe *et al.*, 1984). In addition, *N*-carbamyl-glutamate administration can reduce hyperammonemia by bypassing propionyl-CoA inhibition of *N*-acetyl-glutamate synthetase (O'Connor *et al.*, 1989). In two cases, children with PA who suffered from frequent metabolic decompensation have been treated by liver transplantation (Yorifuji *et al.*, 2000; Kayler *et al.*, 2002). Although liver transplantation had only minimal effects on the levels of propionyl CoA metabolites in the blood, hyperammonemia was corrected and metabolic decompensation was controlled (Yorifuji *et al.*, 2000). More importantly, the growth rate and mental development of these children also improved significantly after transplantation.

Despite the cell autonomous nature of PA, these data suggest that liver correction of PCC can attenuate the systemic and at least some of the most problematic neurologic symptoms of PA. But while liver transplantation can attenuate PA, the danger and immunosuppression intrinsic to this approach mandate that alternate therapies be developed to correct the PA metabolic defect.

A mouse model of PCC deficiency

Dr. Toru Miyazaki's group developed a mouse model of PA by deletion of the α subunit of propionyl CoA carboxylase (PCCA) (Miyazaki *et al.*, 2001). Mice null for PCCA have exceptionally high concentrations of propionic metabolites including serum propionyl-carnitine,

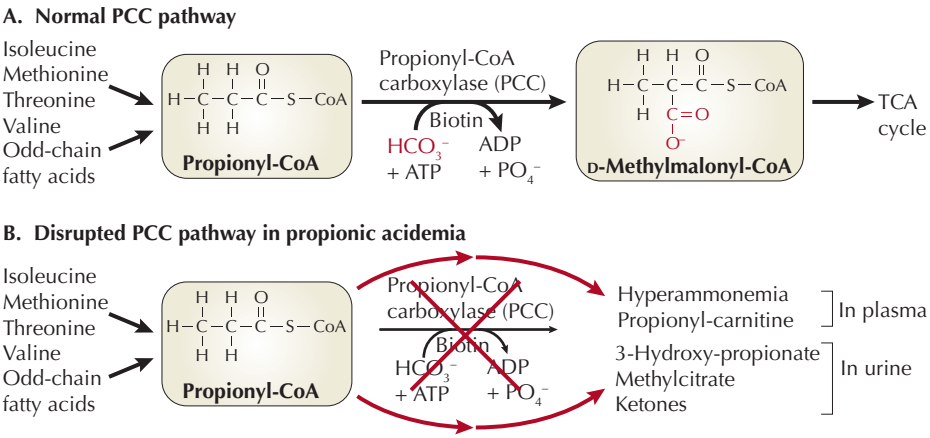

Fig. 1. Simplified propionyl-CoA carboxylase (PCC) metabolic pathways. A) Normal pathway. B) Disrupted pathway when PCC is defective. Aberrant metabolites in the blood and urine are shown in red.

urinary 3-OH-propionate (3-HP) and urinary methylcitrate upon birth and die within 24 hours of birth due to acidosis and poor feeding. These PCCA deficient mice provide a stringent model for PCCA gene therapy where genetic correction must occur before birth or within 36 hours of birth.

Dr. Miyazaki's group generated two additional mouse strains by transgenesis in which the PCCA cDNA is expressed in a liver-specific fashion from the serum amyloid P component (SAP) promoter (Miyazaki *et al.*, 2001). In one PCCA$^{-/-}$ SAP strain, liver-specific PCCA expression remains at 10–20% of normal levels through 8 days after birth, but then rises to near wild-type levels within 3 weeks. This increase in liver-specific PCCA activity decreases blood propionyl-carnitine levels from 17 to 11 µM, but these levels remain substantially higher than the 0.9 µM level in PCCA$^{+/+}$ mice (Miyazaki *et al.*, 2001). These data provide proof of principle for the potential to replace missing PCCA function by liver-directed gene therapy to attenuate the phenotypes associated with PCCA deficiency. This work also provides a low stringency model in which genetic modification is not needed to rescue the animals, but that can be used to test if PCCA gene delivery can further reduce the systemic levels of propionyl-carnitine.

A second strain of PCCA$^{-/-}$ SAP mice also expressed PCCA in a liver-restricted fashion but, unlike the high strain, this expression never rose above 10–20% of wild-type levels (Miyazaki *et al.*, 2001). As a result, these mice survive to weaning, but die before 3 weeks of age due to severe ketonuria and dehydration. This strain provides guidance on the level of genetic correction that will likely be required in any therapy for PA. In this case, long-term expression of PCCA at 10–20% of wild-type levels was insufficient to rescue the animals. These data suggest that any therapy for PA must establish greater than 20% of wild-type expression to rescue the deficiency.

Gene therapy for PA

Dr. Miyazaki's work and past liver transplantations in patients provide excellent proof of principle for the possibility of performing gene therapy in the liver to at least mitigate the symptoms of PA. This is fortuitous, as most gene therapy vectors preferentially deliver genes into this organ. The liver is a logical target for gene therapy for PA, because the liver is thought to be the 'sink' for much of the metabolism in the propionic pathway. While a number of useful gene therapy vectors are being developed for liver gene therapy, very few can express the massive levels of transgene that will likely be needed to correct PA deficiency even in a mouse. While non-viral vectors may be potentially safer gene delivery vehicles than viral gene therapy vectors, few or none currently can drive the high level correction that will be needed for PA. Given this, we will discuss two promising viral vectors for PA gene therapy and look forward to the development of safer and more effective non-viral and viral vectors for PA treatment.

Adenoviral vectors for liver-directed gene therapy

Adenoviral (Ad) vectors are arguably the most robust vectors for mediating high level gene expression in the liver. Several studies have shown that intravenous injection of Ad vectors into mice and primates results in 70% of total gene delivery occurring in the liver (Tang *et al.*, 1994; Huard *et al.*, 1995). The high level of gene delivery occurs predominantly by transduction of hepatocytes (Hegenbarth *et al.*, 2000). Ad vectors are able to transduce a higher fraction of liver hepatocytes with levels of modification occurring in nearly 100% of hepatocytes (Yang *et al.*, 1996a; Morral *et al.*, 1997). Few other vectors have been shown to mediate the same robust level of liver transgene expression as Ad vectors. For example, liver transduction by Ad vectors expressing α1-antitrypsin produces supraphysiologic levels of 6 mg/ml of α1-antitrypsin in the blood of mice (Morral *et al.*, 1998). Or in other words, modification of the liver with Ad vectors was able to produce this transgene product at such high levels that α1-antitrypsin became the second most abundant protein in the blood of the animals. Similarly, Ad vector delivery of the apolipoprotein A-1 gene to the liver also produces supraphysiologic levels of this transgene in Apo-E-deficient mice (Pastore *et al.*, 2004).

From these observations, it is possible to argue that the supraphysiologic expression ability of Ad vectors may produce sufficient PCCA protein in the liver to further reduce propionyl-carnitine levels in PCCA$^{-/-}$ SAP high mice. Likewise, Ad vectors should be able to produce the greater than 20% of wild-type PCCA expression needed to correct the PA phenotype in PCCA$^{-/-}$ mice.

While Ad vectors are potent for mediating gene delivery, they also have the reputation for being one of the most immunogenic vectors for gene therapy (Fields *et al.*, 2000). Both innate and adaptive immune responses can be elicited Ad capsid proteins and against the transgene protein itself to attenuate gene delivery and expression. Innate immune responses occur in a dose-dependent fashion in mice, non-human primates and humans after intravenous injection of Ad vectors. Uptake of Ad virions by immune and non-immune cells precipitates the release of massive amounts of inflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor (TNF)-α within 3 to 24 hours of intravenous injection (reviewed in Liu and Muruve, 2003). These events produced by intravenous administration of large doses (e.g. 10^{13} virus particles/kg) can lead to lethal events in non-human primates (Brunetti-Pierri *et al.*, 2004). Innate responses to Ad vectors also likely played a significant role in the unfortunate death of Jessie Gelsinger in the ornithine transcarbamylase (OTC) gene therapy trial (Marshall, 1999).

Helper-dependent adenoviral (HD-Ad) vectors

Early work with first-generation Ad vectors for liver-directed gene therapy showed that transient gene expression was in part due to cytotoxic T-cell responses directed against Ad antigens that were expressed in a leaky fashion off of the vector genome (Yang *et al.*, 1996b). To reduce their immunogencity, helper-dependent Ad (HD-Ad) vectors were developed in which all viral genes are deleted from the vector (Mitani *et al.*, 1995; Clemens *et al.*, 1996; Fisher *et al.*, 1996). These HD-Ad vectors mediate substantially reduced levels of hepatotoxicity after intravenous injection than first generation Ad vectors and showed markedly prolonged transgene expression in mice and in baboons due to ablation of cellular immune responses against viral antigens in vector-modified cells (Chen *et al.*, 1997; Morral *et al.*, 1998; Morral *et al.*, 1999). Based on their lower immunogenicity and liver toxicity, recent work has shown that HD-Ad vectors can mediate long-lasting and life-long genetic correction of liver diseases in mice. For example, HD-Ad has been used to deliver the very low-density lipoprotein receptor into mice deficient for the low-density lipoprotein receptor to mediate stable correction for 6 months (Oka *et al.*, 2001). Similarly, HD-Ad vectors expressing apolipoprotein E (Apo-E) can mediate life-long correction in ApoE deficient mice (e.g. 2.5 years; Kim *et al.*, 2001). HD-Ad vectors have also mediated long-term correction of the metabolic disorder due to ornithine transcarbamylase (OTC) deficiency (Mian *et al.*, 2004). Given that gene therapy for OTC requires its gene to be expressed at levels greater than 10–20% of wild-type expression, these data suggest that these safer HD-Ad vectors may have utility for treating PA.

Reducing the toxicity of Ad vectors using polyethylene glycol

Polyethylene glycol (PEG) is a clinically-approved conjugation agent used to improve the pharmacokinetics of a variety of protein therapeutics. In these applications, the hydrophilic PEG molecule is cross-linked to the therapeutic agent to 'shield' or reduce interactions of it with proteins and cells that would normally decrease the therapeutic's interactions with its target. PEG has also been applied to improve the pharmacology of Ad vector. PEG molecules bearing reactive groups are chemically conjugated to free amine groups on the virion surface. By this approach, as many as 15,000 PEG molecules can be added to the virion surface to reduce its interactions with a variety of biomolecules and cells. Previous work has shown the utility of this approach to protect Ad vectors pre-existing neutralizing antibodies to allow multiple administration into immune recipients and has also demonstrated that PEGylation reduces the production of new antibody and cellular immune responses against Ad proteins (Croyle *et al.*, 2001).

Based on PEG's shielding functions, we have tested its ability to reduce *in vivo* non-specific interactions that are involved with provoking the problematic responses related to this vector. First-generation Ad (FG-Ad) and HD-Ad vectors were PEGylated with approximately 15,000 PEG molecules coated each virion. Notably, PEGylation reduced innate immune responses directed against Ad vectors, as evidenced by 75% reduction in IL-6 responses at 6 hours and a 90% reduction in cumulative IL-6 over 48 hours (Mok and Barry, 2005). While PEG itself did not reduce liver damage after 5 days for FG-Ad vectors, PEGylated HD-Ad vectors produced no liver damage. The effects of PEGylation on reducing innate immune responses appears to be due in part to the ability of this shielding agent to reduce non-specific uptake of the vector by antigen-presenting cells like macrophages and Kupffer cells (Mok and Barry, 2005). Our more recent work has shown that PEGylation also blocks binding of Ad to platelets, red blood cells, and endothelial cells, and also blunts Ad-induced thrombocytopenia, disseminated intravascular coagulation (DIC), and splenomegaly (unpublished observations). These data suggest that this simple vector modification may have utility to improve the safety of these robust Ad vectors for PA gene therapy.

Adeno-associated virus vectors

Adeno-associated virus (AAV) vectors also hold promise for liver gene therapy of PA. AAV vectors can deliver genes episomally to cells, but also integrate genes into host chromosomes to a low degree. So, AAV vectors have the benefits of both non-integrating Ad vectors and integrating retroviral vectors, but also perhaps their types of problems—loss of transgene during cell division by episomal vector and potential for oncogenic activation for integrating vector. AAV-2 vectors have demonstrated successful and sustained liver gene therapy of factor IX deficiency in mouse models (Snyder *et al.*, 1997; Wang *et al.*, 1999). One primary advantage that AAV vectors have over Ad vectors is that they appear to be substantially less immunogenic and cause lower side effects in animal models (Jooss *et al.*, 1998; Fields *et al.*, 2000). AAV-2 vectors pseudotyped with AAV-8 capsid (AAV-2/8) have recently been shown to transduce 80 times higher transgene expression from the liver than AAV-2 vectors and 10-fold higher expression than vectors pseudotyped with the capsids from AAV-5 and AAV-7 (Gao *et al.*, 2002). When applied in LDL-receptor-deficient mice, AAV-2/8 mediated near complete normalization of serum lipids and protected from atherosclerosis (Lebherz *et al.*, 2004). These data suggest that AAV vectors may provide sufficient genetic correction in the liver to produce the greater than 20% of wild-type PCCA expression needed to rescue $PCCA^{-/-}$ mice from lethal PA.

Although these data suggest the potential of AAV, this vector integrates to a low level into the genome, increasing the possibility of oncogenesis (Hacein-Bey-Abina *et al.*, 2003a; Hacein-Bey-Abina *et al.*, 2003b) and chromosome rearrangements (Miller *et al.*, 2002). In addition, recent human trials have observed immune-mediated rejection of vector-modified cells similar to that observed with FG-Ad vectors (Manno *et al.*, 2006). These data suggest AAV may confront problems with pre-existing or vector-induced immunity in a manner similar to that confronted by Ad vectors. Nonetheless, AAV does not generate the same innate immune responses that Ad vectors provoke and may ultimately be a safer gene therapy vector for PA than Ad vectors.

Where should gene therapy for PA be delivered?

The liver is a logical target for gene therapy interventions given its pivotal role in metabolism. However, it is unclear if it is the only site of genetic intervention that can mitigate the symptoms of PA. While much is known about the genetics and disease symptoms of PA, little data can be found in the literature regarding the distribution of PCCA protein in different tissues. We thought this was an important question, as the level of protein may explain some of the tissue damage and symptoms due to loss of PCCA. Likewise, knowing where PCCA is expressed might better guide how transplantation and gene therapies need to be applied. For example, one might predict that the liver expresses the highest level of PCCA given its role in metabolizing excess amino acids and fatty acids. Conversely, one might predict that the basal ganglia might express lower amounts of PCCA, as many of the symptoms of the disease are manifested in those brain structures.

Given these issues, we generated antibodies against both mouse and human PCCA and performed a screen for PCCA protein in mouse and human tissue. While we expected PCCA to be either ubiquitously expressed or expressed at highest levels in the liver, to our surprise we observed a marked variation in amount of PCCA in different tissues. In both mouse and human tissues, the kidney appeared to have the highest level of PCCA protein, higher than in the liver (unpublished observation). In contrast, in the brain, PCCA was undetected in mouse (but not necessarily zero), and was detectable, but at low levels in the human samples. These data suggest that PCCA is not ubiquitously expressed at high levels and that the kidney may play a significant role in elimination. While the kidney had higher PCCA levels (even if normalized to tissue protein content), it should be noted that the liver likely still plays a more important role than the kidney in the disease, given this organ's substantially higher mass and other metabolic flux.

Prospects

Dr. Miyazaki's work has provided excellent proof of principle for the possibility of performing liver gene therapy to at least mitigate the symptoms of PA. While promising, his group also pointed out that this approach does not genetically correct all of the cells of the body. So, if there is cell autonomous toxicity within each cell, liver gene therapy cannot correct every aspect of the disease. Our work with the PA null mice has revealed some unreported phenotypic changes in the mice that might speak to this issue. While surviving $PCCA^{-/-}$ SAP mice are reported to be essentially normal, we have found that these mice have some growth delays and breed poorly (unpublished observation). This suggests that the level of liver genetic correction achieved in these mice may be insufficient to fully counter the systemic effects of the disease. Indeed, even 50% of normal PCCA expression may be somewhat limiting, as mice heterozygous for

the PCCA gene have subtle, but detectable behavioral phenotypes (unpublished observation). If this is the case in both mice and humans, liver correction may need to be supplemented with gene therapy in the brain to correct cell autonomous effects. Nonetheless, liver correction by itself appears to markedly improve many of the symptoms of the disease in mice and in humans. Therefore, there is still great merit in striving to achieve liver (or other site) gene therapy to improve symptoms without necessarily correcting the PCC genes throughout the body. Once liver gene therapy for PA is achieved, we can explore the cost/benefit of performing more systemic gene therapy for the disease.

Additional issues in treating PA by gene therapy will be deciding when to intervene and developing approaches that can mediate persistent genetic correction. The Ad and AAV vectors we are testing can mediate sustained genetic correction in adult mice over a few years, but the short life span of the animals makes it difficult to know exactly how long this could persist. In contrast to adults, if we want to intervene in a newborn to stave off the early side effects, one must confront the fact that the liver will be growing and that this may dilute out the genetic correction over time. So, one may need to intervene with a series of treatments to keep up with liver expansion.

Alternately one may recruit the use of more stable gene therapy vectors. Vectors like lentiviruses that physically integrate genes into chromosomes would be an excellent choice for this application. However, the recent observation of oncogenesis in a subset of children who received integrating retroviruses in the French severe combined immunodeficiency (SCID) human trial (Hacein-Bey-Abina *et al.*, 2003b) make their use for PA less attractive at this moment. It should be noted that this side effect in the SCID trial is thought to be due in part to the fact that this gene therapy was delivering a potent growth factor signaling protein into the cells of the patient. As some oncogenes are also signaling molecules, it is thought that the particular gene used for gene therapy in this case may have delivered the first 'hit' for cancer progression, and that this may have set the stage for the observed oncogenesis. In contrast, PCC is a mitochondrial metabolic enzyme that is unlikely to stimulate uncontrolled cell growth, so delivery by an integrating vector may not produce similar side effects. Work is underway to make these integrating vectors safer and to make other episomal vectors more persistent. It is likely that these vectors will provide new tools for PA gene therapy.

While genetic correction in a growing mouse or human presents a more challenging environment to gene therapy, intervening as early as possible in a PA patient may also have some immunological benefits. If PA children have mutations that affect the immunogenicity of the protein or that delete sections of the protein, this may make later therapy more difficult. In these cases, if the correct gene is successfully delivered, expression of the previously missing protein sequences in PA may provoke the immune system to consider them as foreign, and these cells will be killed by the immune system. If observed, one may have to apply immunesuppression similar to that used in liver transplantation itself. Conversely, if the PA gene (or protein) is delivered to children in the neonatal period, this may have the benefit to tolerize their immune system to the protein. This could provide a future window of opportunity for subsequent, more effective, gene or stem cell therapies to be performed without the danger of immune attack. This will likely not be necessary in patients with single point mutations, but may be needed if additional research finds splicing mutations that delete sections of the protein (Desviat *et al.*, 2006).

While many issues still challenge gene therapy for PA, an increasing number of laboratories are working to take these first steps towards providing treatments to reduce the symptoms of the disease. Preliminary data from our laboratory indicates that delivery of Ad vectors expressing PCCA into newborn PCCA null mice within hours of birth can extend their lifespan beyond their normal death at 24 hours (unpublished observation). These data provide hope that gene therapy can be used to treat PA. Work is underway to validate these results and optimize these approaches to provide safe and persistent genetic correction.

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FUNCTIONAL ANALYSIS OF SPLICING MUTATIONS CAUSING ORGANIC ACIDEMIAS

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Mutations affecting the splicing of pre-mRNA are one of the most common causes of disease and may have different functional consequences depending on the nucleotides affected and the local sequence context. We have investigated the transcriptional profile of different splicing mutations identified in patients with propionic acidemia, isolated methylmalonic acidemia or methylcrotonylglycinuria with the aim of clarifying their functional effect and involvement in the disease phenotype and to serve as basis for the development of novel pharmacological or molecular therapies aimed at the correction of erroneous splicing. In some cases, we identified detectable levels of normally spliced transcripts, which may be increased using different compounds that act as transcriptional activators such as aclarubucin, valproic acid or kinetin. In addition, we have identified aberrant inclusions of pseudoexonic sequences, which may be targeted with antisense oligonucleotides to correct the splicing process.

In the past few years, the field of genetic diseases has given special attention to research on mutations affecting splicing, which generally account for 10–30% of the total mutant alleles (Krawczak *et al.*, 2000). In eukaryotes, protein-coding genes have large non-coding sequences (introns) separating the sequences that code for the protein (exons), all of which are transcribed into the pre-mRNA. The introns are removed in a highly specific manner during the splicing process. To correctly identify and join together the exons, there are conserved motifs in or near the intron-exon boundaries that act as splicing signals: the 3′ and 5′ splice sites, a polypyrimidine tract and the branch site. In practically all cases, the 5′ splice site includes a guanine/thymidine (GT; guanine/uracyl (GU) in the mRNA) dinucleotide, whereas the 3′ splice site has an adenine/guanine (AG) dinucleotide. In addition to these conserved sequences, there are regulatory elements (splicing enhancers or silencers) located in exons or introns needed to allow normal splicing of some exonic sequences (FIG. 1). Mutations in all these sequences may disrupt splicing (Blencowe, 2000).

The two most common consequences of a splicing mutation are exon skipping or cryptic splice site activation, resulting in the insertion or deletion of nucleotides in the mature mRNA (Faustino and Cooper, 2003). The mechanism by which one or other is chosen for a given mutation in a specific gene context is not yet fully elucidated, but clearly depends on the local sequence, which makes it practically impossible to predict the effect of a given splicing mutation. It is therefore necessary to analyze the transcriptional profile associated with each splicing mutation, either in patients' cells or by the use of minigenes that contain the genomic sequences to be analyzed in vectors specifically designed to allow exon splicing. These vectors with normal or mutant sequences are transfected into established cell lines to characterize the pathological effect of each mutation (Cooper, 2005). The analyses require RT-PCR and qRT-PCR (quantitative RT-PCR by real-time PCR methodologies) using specific primers designed to amplify the corresponding transcripts, and cloning to rescue minor transcripts.

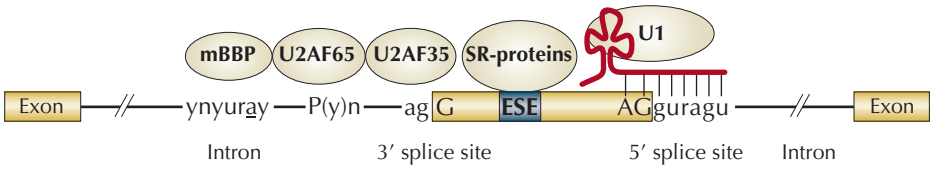

FIGURE 1. Schematic representation of the cis-sequences and trans-acting factors involved in splicing. U1 SnRNP binds to the 5′ splice site, and different proteins bind to the 3′ splice site (U2AF35 to the invariant AG, U2AF65 to the polypyrimidine tract and mBBP to the branch point sequence). Less conserved sequences such as exonic splice enhancers (ESE) are recognized by SR proteins.

Most of the splicing mutations result in a frameshift in the coding sequence and the introduction of a premature termination codon (PTC) and are therefore substrates of nonsense-mediated decay (NMD). NMD actively degrades PTC-containing mRNAs, preventing the generation of truncated proteins potentially toxic to cells (Maquat, 2004).

Some splicing mutations completely abolish exon recognition and result in complete absence of correctly spliced transcripts. Others generate both aberrantly and correctly spliced transcripts, the level of which may vary among patients and tissues. Moreover, the levels of aberrant and normal transcripts may be associated with specific disease phenotypes; the higher the levels of normal transcripts, the milder the phenotypes (Clavero *et al.*, 2004; Lualdi *et al.*, 2006). These observations have raised the possibility that splicing modulation may be a modifier of disease severity in patients carrying splicing mutations that generate normal transcripts (Nissim-Rafinia *et al.*, 2004).

Splicing modulation can be achieved by overexpression of specific splicing factors or by use of drugs such as aclarubicin, sodium butyrate, kinetin or valproic acid, which have been proven useful in the reversion of aberrant splicing (Andreassi *et al.*, 2001; Nissim-Rafinia *et al.*, 2004). These compounds act as transcriptional activators due to their ability to inhibit histone deacetylase, and can increase the levels of correctly spliced transcripts, improving the prognosis of disease caused by splicing mutations.

Another promising splicing therapy, already in clinical trials, is the use of antisense oligonucleotides (AONS) directed at the prevention of the inclusion of aberrant sequences in the mature mRNA, such as intronic sequences (pseudoexons), which are recognized as exons due to mutations activating cryptic splice sites. AONs can modulate the splicing pattern by steric hindrance of the binding of the splicing apparatus to the selected sequences, thus forcing the machinery to use the natural sites. Antisense therapy modulating splicing has been used successfully in cystic fibrosis and Duchenne muscular dystrophy. In the latter case, it is already in use in patients (Takeshima *et al.*, 2006).

Our group is involved in the diagnosis of and research on different inborn errors of metabolism (IEM), in particular, three organic acidurias: propionic acidemia (PA), methylmalonic acidemia (MMA) and methylcrotonylglycinuria (MCG). PA is caused by defects in the *PCCA* or *PCCB* gene, which code for the two subunits of the enzyme propionyl-CoA carboxylase. MMA is linked to mutations in the genes *MUT*, *MMAA* or *MMAB*, which code for the enzyme methylmalonyl-CoA mutase and for enzymes involved in the synthesis of the active form of the cofactor adenosylcobalamin, respectively. MCG results from mutations in the genes *MCCA* or *MCCB*, which code for both subunits of the enzyme methylcrotonyl-CoA carboxylase. Our research in these disorders has covered different aspects, from gene identification and characterization, mutation analysis and functional expression of variant alleles, molecular basis of the pathophysiology and investigation on novel pharmacological and genetic mutation-specific therapies.

In this work, we review our results on the functional analysis of splicing mutations in organic acidemias. We summarise the results of the analysis of the transcriptional profile of mutations affecting splice sites by RT-PCR and qRT-PCR using patients' fibroblasts and minigenes. We have characterized some mutations with detectable levels of correctly spliced mRNA, which may be candidates for splicing therapeutics such as those described above. In addition, we have recently identified several aberrant inclusions of pseudoexonic sequences, candidates to antisense therapeutics.

Intronic splicing defects

TABLE 1 shows the summary of results obtained studying intronic splicing mutations to determine the levels of normally spliced transcripts. These mutations are severe, involving exon skipping or activation of new intronic and exonic cryptic splice sites. In some cases, further analysis by minigene cell expression systems and/or qRT-PCR analysis revealed the presence of different levels of normal transcripts (Clavero *et al.*, 2004; Desviat *et al.*, 2006), which identifies them as candidates for splicing therapies aimed at raising these levels. Therapeutic modification of the transcript profile has been described in other genetic diseases using drugs such as aclarubicin (Andreassi *et al.*, 2001), valproic acid (Brichta *et al.*, 2003) or kinetin (Slaugenhaupt *et al.*, 2004). Some mutations are further described below.

TABLE 1. Intronic sequence changes affecting mRNA splicing found in genes defective in organic acidemias

Mutation	Gene	Disease	Splice analysis methods	Normal transcripts	Reference
IVS2-2A>G	MCCA	MCG	Minigenes	Yes	(Rincon *et al.*, 2007a)
IVS13+3A>G	PCCA	PA	qRT-PCR (fibroblasts)	2–3%	(Desviat *et al.*, 2006)
IVS21+3del4	PCCA	PA	qRT-PCR (fibroblasts)	6.3%	(Clavero *et al.*, 2004)
IVS22-2A>g	PCCA	PA	qRT-PCR (fibroblasts, minigenes)	6–16%	(Clavero *et al.*, 2004)
IVS1+2T>C	PCCB	PA	RT & real time PCR and melting probe analysis (fibroblasts)	Not detected	(Desviat et al., 2006)
IVS10-11del6	PCCB	PA	RT & real time PCR and melting probe analysis (fibroblasts)	Yes	(Clavero *et al.*, 2004)
IVS14+2T>C	PCCB	PA	qRT-PCR (fibroblasts)	0.1%	(Desviat *et al.*, 2006)
c.291-1G>A	MMAB	MMA	qRT-PCR (fibroblasts)	Not detected	(Rincon *et al.*, 2005)

Mutation IVS21+3del4 (c.1899+3del4) in the *PCCA* gene lowers the splicing score of the 5′ splice donor site of exon results in exon 21 skipping, corresponding to an in-frame deletion of 54 base pairs (bp; 18 amino acids), which are associated with a total absence of enzyme activity and an accelerated turnover of the mutant protein in mitochondria. These results contrast sharply with the mild phenotypic expression of the disease (late onset and near-normal development) in a homozygous patient. To test if some normally spliced transcript was present in the patient's cells, which could explain the mild phenotype, we performed RT-PCR analysis with exon-specific primers, which would selectively amplify exon 21 containing transcripts even if they were present in very low abundance. Some amount of normally spliced transcript, as confirmed by sequence analysis, was indeed detectable in these conditions by ethidium bromide staining. The exact levels of normal transcript were quantified by real time PCR and found to be ~3% (Clavero *et al.*, 2004).

Mutation IVS22-2A>G (c.2041-2A>G) in the *PCCA* gene affects the invariant AG dinucleotide at the 3′ acceptor splice site in exon 23 of the PCCA gene, causing exon 23 skipping. Exon 23 contains the conserved A-M-K-M motif responsible for biotin binding, and absence of this sequence results in a non-functional protein, but the phenotype of a homozygous patient was moderate. Using specific primers, normal transcript was quantified in patients' fibroblasts corresponding to 16 ±6.4% compared to the amount obtained in normal fibroblasts (Fig. 2). This result was reproduced using minigene constructs (Clavero *et al.*, 2004).

Mutation IVS13+3A>G (c.1209+3A>G) in the *PCCA* gene is a transition affecting the 5′ donor splice site of exon 13. At the cDNA level, the skipping of exon 13 is observed, which predictably gives rise to a protein with an internal deletion of 48 aminoacids. This change has been detected in several patients of common geographical origin, one of them so far asymptomatic after being detected in a neonatal screening program. We investigated the possibility of the presence of some normal transcript in patients´ fibroblasts. For that purpose, we isolated RNA from fibroblasts of patients homozygous for this change and quantified the presence of exon 13-containing (i.e. normal) transcripts by real- time PCR using two methodologies: i) amplification using an oligonucleotide hybridizing to exon 13 and the double helix-specific dye SYBR Green I; ii) amplification and detection with a Taqman probe specific for the cDNA region corresponding to the exon 13-exon 14 junction. Using the SYBR Green I methodology, we could not detect any normal transcript compared with control samples. Using a Taqman probe specific for exon13-exon14 containing sequences, we could detect 1.8%-2.7% of normal transcripts in fibroblasts (Desviat *et al.*, 2006).

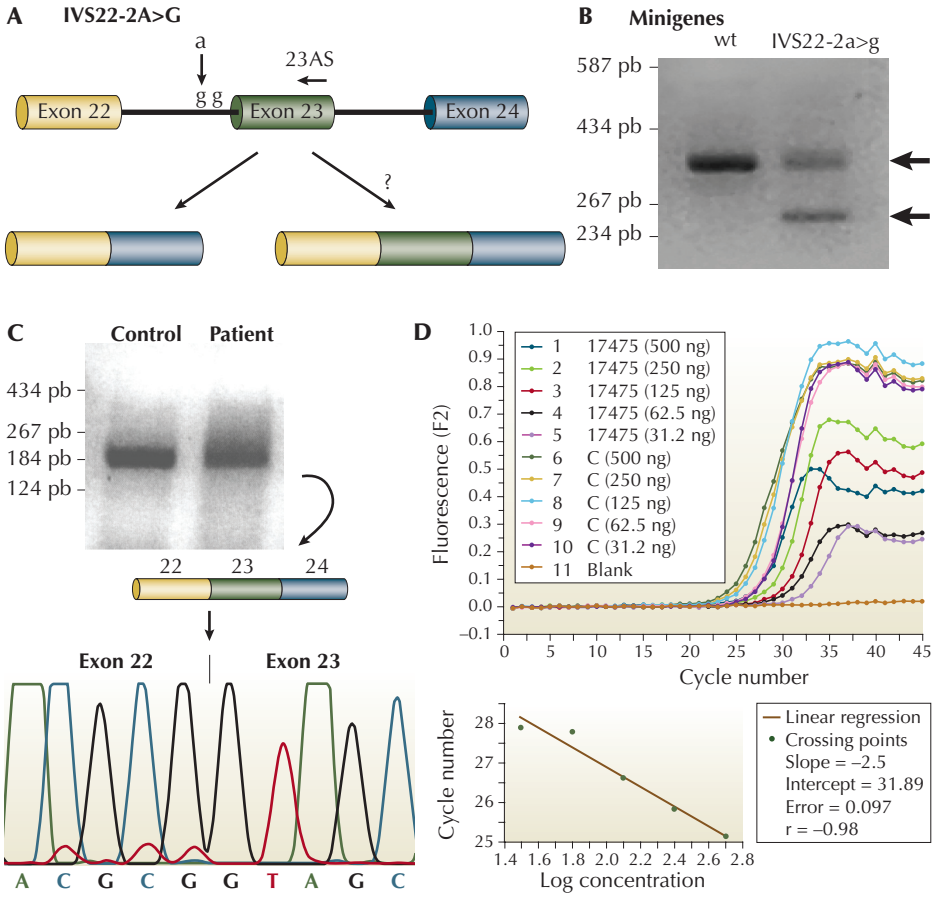

Figure 2. Analysis of the effect of the IVS22-2A>G mutation in the PCCA gene. (A) Schematic representation, (B) RT-PCR analysis in COS cells with minigene constructs, (C) RT-PCR analysis in patient's fibroblasts and (D) qRT-PCR analysis in patient's fibroblasts. In minigene analysis, the smaller band in the patient's sample corresponds to the exon skipped product not present in the wild type (wt) construct. In patient's fibroblasts, PCR was performed using primer 23AS (denoted by arrows) hybridizing to the exon that is skipped as a consequence of the splicing mutation. The sequence files show the relevant exon junctions in the amplified band from patient, confirming correct splicing. qRT-pCR analysis indicated there is 16% of correctly spliced transcripts compared with a control fibroblast. (Figure reproduced from Clavero *et al.*, 2004).

Exonic splicing defects affecting U1snRNP binding

The last exonic nucleotides are included in the 5′ donor splice site (Fig. 1) and mutations affecting them may be classified erroneously as missense mutations, if their effect on splicing is not analyzed. This effect depends on the sequence context and the strength of base pairing with the RNA component of U1snRNP, which binds to this site during splicing. We have investigated the transcriptional profile of different base substitutions in the last exonic nucleotides identified in different genes to characterize precisely their effect (Perez *et al.*, 2003; Martinez *et al.*, 2005). The base pairing of the mutant sequences with U1snRNP is shown in Table 2. In some cases, complementation with modified U1snRNAs can provide clues to a specific therapeutic strategy to correct splicing defects at the 5′ splice site (Susani *et al.*, 2004) .

The c.653A>G mutation in the *PCCB* gene was initially classified as missense, causing the change K218R which was expressed in vitro and found to be associated with high (70%) residual activity (Perez-Cerda *et al.*, 2003). However, subsequent mRNA analysis revealed that the mutation, located in the last codon of exon 6 of the *PCCB* gene, causes aberrant splicing, activating a cryptic splice acceptor site within exon 6. The effect is an in-frame deletion of six nucleotides in the mRNA, which would result in a protein with two amino acids deleted (V217–K218) with null activity. This mutation is present in heterozygous fashion in a patient carrying

TABLE 2. Exonic sequences changes found in MMA and PA genes affecting U1snRNP binding

Mutation (gene)	5′ splice site score[a] wt→mutant	U1snRNP binding[b]	Major effect	Reference
c.763G>A (PCCB)	0.95→0.63	AA]gugagagg	100% correctly spliced with missense change G255S	–
c.653A>G (PCCB)	1→0.99 new donor site: 1	GG]guaagaaa	Cryptic splice site activation; 6 nt deletion (delV217-K218)	(Clavero *et al.*, 2004)
c.1808G>A (MUT)	0.96→0.53	AA]guaagaaa	Cryptic splice site activation, 62nt deletion (V583fs)	(Martinez *et al.*, 2005)
c.562G>C (MMAA)	1→0.98	AC]gucagucu	100% correctly spliced with missense change G188R	(Rincon *et al.*, 2007a)
c.733G>A (MMAA)	0.96→0.53	UA]gugagugu	Exon 4 skipping and minor levels with missense change G245S	(Rincon *et al.*, 2007a)
c.584G>A (MMAB)	0.99→0.6	CA]guaagagg	Exon 7 skipping.	(Rincon *et al.*, 2007a)

[a]BDGP software (www.fruitfly.org/seq_tools/splice.html)
[b]Underlined bases represent base pair matches with U1snRNA. U1 consensus sequence AG]guragu

R165Q on the other allele, a mutation that also has null residual activity in expression analysis (Perez-Cerda *et al.*, 2003). The patient has a mild phenotype with normal development.

In view of this, we decided to test the possibility that the c.653A>G mutation also produced some amount of normally spliced product, which would result in a protein with the K218R missense change with partial residual activity that would account for the mild phenotype in the patient. The mutation was tested in an in vitro splicing assay using minigenes and was found to result in the 6-nucleotide deletion in the mRNA through activation of the cryptic splice site in exon 6, as observed in patient cells.

As we could not discard the presence of normal spliced product in very low amounts not detectable by conventional RT-PCR and sequence analysis, we designed allele-specific hybridization probes to use with real-time fluorescent PCR methodology analysing patient's cells transcripts. The results confirmed the absence of normally spliced transcript in the patient's cells indicating that the mutation completely abolishes the recognition of the mutant 5′ splice donor site (Clavero *et al.*, 2004).

In contrast, the c.562G>C mutation in the *MMAA* gene and the c.763G>A mutation in the *PCCB* gene produce mainly molecules correctly processed bearing the missense changes G188R and G255S respectively, an observation that correlates with the very slight decrease in splicing score.

The two other mutations tested, c.733G>A in the *MMAA* gene and c.584G>A in the *MMAB* gene cause skipping of the corresponding exon generating a frameshift and a premature termination codon in the coding sequence. In the first case, we could detect cDNA molecules of complete length bearing the missense mutation G245S by subcloning the fragments obtained by RT-PCR in patient's fibroblasts and sequencing. In the latter case, minor but detectable amounts of correctly spliced transcripts were obtained in minigenes transfected in some cell types (HEK or COS) and not in others (hepatoma), indicating tissue specific difference in splicing factors that may modify the phenotype (Rincon *et al.*, 2007a).

Pseudoexon splicing defects

Recently, we have found deep intronic mutations leading to the insertion of a pseudoexon or cryptic exon in the mRNA of the genes defective in MMA and PA. Exonization of intronic sequences has been described in other genetic diseases and represents a rare phenomenon (Tuffery-Giraud *et al.*, 2003; Raponi *et al.*, 2006). Nevertheless, the true prevalence of these mutations is underestimated as few laboratories analyze intron sequences so far away from coding regions, and the corresponding transcripts are usually substrate of the NMD pathway. We have identified this kind of mutations in homozygous fashion or combined with other NMD substrate mutations in *PCCA*, *PCCB*, *MUT* and *MMAB* affected patients. We have identified a 72 bp insertion between exons 6 and 7 in the *PCCB* gene (r.654ins72bp), and an 84 bp insertion between exon 13 and exon 14 in *PCCA* (r.1209ins84), both in homozygous fashion, and two insertions in heterozygous fashion: a 76 bp insertion (r.1957ins76) between exons 11 and 12 in the MUT gene and a 97 bp insertion between exons 6 and 7 in the MMAB gene (r.518ins97). The intronic mutations found in the genomic DNA activate cryptic splice sites or eliminate or generate consensus binding motifs for splicing factors such as SC35, SRp55 or SRp40, which most likely favor the intronic inclusion (Rincon *et al.*, 2007b). To eliminate the intronic insertions, we are investigating the possibility of blocking the pseudoexons splice sites using antisense morpholino oligonucleotides to restore correct splicing in PA and MMA affected genes. Therapeutic modifications of the splicing pattern with antisense oligos have been shown in other genetic diseases such as β-thalassemia/HbE disorder (Suwanmanee *et al.*, 2002), cystic fibrosis (Friedman *et al.*, 1999), Duchenne muscular dystrophy (Aartsma-Rus *et al.*, 2003), ocular albinism Type I (Vetrini *et al.*, 2006) and others.

Concluding remarks

These results reflect the different consequences derived from splicing mutations, which, in turn, can be influenced by complex networks of splicing factors differentially expressed in each tissue and/or individual, thus resulting in phenotypic variability. Investigation of the functional consequences of splicing mutations using different approaches is essential for the development of novel pharmacological or molecular therapies aimed at the correction of erroneous splicing. The blocking of aberrant splicing with antisense oligonucleotides and use of drugs or splicing factors that modulate splicing is the aim of our present research.

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