MINI-REVIEW

Cell Culture Studies on Patients with Mitochondrial Diseases: Molecular Defects in Pyruvate Dehydrogenase

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Abstract

There is a group of inborn errors of metabolism that result in the condition of chronic lacticacidemia of childhood. Nearly all of the defects that can be identified occur in mitochondrial proteins, and can be demonstrated in cultured skin fibroblasts from the patients concerned. One approach to the diagnosis of these defects involves a simple incubation of the fibroblast culture with glucose-containing medium followed by the measurement of accumulated lactate and pyruvate. The total amounts of lactate and pyruvate and the ratio between them is different in cells from patients with defects in the pyruvate dehydrogenase complex or the respiratory chain. Measurement of $1\text{-}^{14}C$-pyruvate oxidation to $^{14}CO_2$ can also reveal defective oxidative metabolism. Localization of the defects can be achieved using individual assays for the enzymes concerned. The clinical sequelae of the different defects is discussed.

Key Words: Chronic lacticacidemia; mitochondrial proteins; skin fibroblasts.

Introduction

For many years our group has worked on the inborn errors of metabolism that result in the condition of chronic lacticacidemia in childhood. These studies have resulted in the clinical description of large cohorts of patients with defects in the pyruvate dehydrogenase complex (Robinson *et al.*, 1987a), pyruvate carboxylase (Robinson *et al.*, 1987b), and the respiratory chain defects (Robinson *et al.*, 1987c). These studies have been carried out

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almost exclusively on skin fibroblast cultures taken from the patients concerned. This article will examine the approaches used in the use of cell culture to diagnose mitochondrial defects.

Approach to Diagnosis of Defects

Cultured skin fibroblasts, as with many other types of cultured cells, derive a large amount of ATP from glycolysis and therefore are not an ideal model for studying defects that affect mitochondrial energy production. In culture medium which contains 5 mM glucose, fibroblasts wil convert 84% of the glucose to lactic and pyruvic acids which accumulate in the medium. Only when glucose in the culture medium is exhausted do the cells become more oxidative and the lactate and pyruvate in the medium are consumed (McKay *et al.*, 1983). Skin fibroblasts which are deprived of culture medium also have a store of glycogen which can sustain them for a number of hours without added nutrients.

We have exploited some of the peculiarities of fibroblast metabolism to assist in the location of defective areas of metabolism in patient-derived cultures.

In normal fibroblasts, as in other cells, the lactate dehydrogenase reaction is at equilibrium such that

$$\frac{\text{(lactate)}}{\text{(pyruvate)}} \times K_{\text{LDH}} = \frac{\text{(NADH)}}{\text{(NAD}^+)}$$

where K_{LDH} is the equilibrium constant of the LDH reaction

$$1.11 \times 10^{-4} \times \tfrac{2.5}{1} = 27.75 \times 10^{-4} = \text{NADH/NAD}$$

$$360 = \text{NAD/NADH}$$

Since the ratio of lactate to pyruvate that accumulates after incubation of the cells with glucose is about 25 to 1, this gives a calculated value for the cytosolic NAD/NADH of 360, a value similar to the ratio seen in other mammalian cells. An equilibrium is also maintained between the cytosolic pyridine nucleotide redox state and the intramitochondrial pyridine nucleotide redox state. These couples are linked by the glutamate/aspartate shuttle system shown in Fig. 1 such that the mitochondrial NAD/NADH ratio is much more in favor of the reduced form being between 10 and 30 : 1 (Krebs, 1973). The difference in the cytosolic and mitochondrial redox states is maintained by the electrogenic expulsion of aspartate from the mitochondrial to the cytosolic compartment (Williamson *et al.*, 1973). The $\text{NAD}_\text{m}/\text{NADH}_\text{m}$ in turn is in equilibrium with the oxidized–reduced cytochrome c couple in
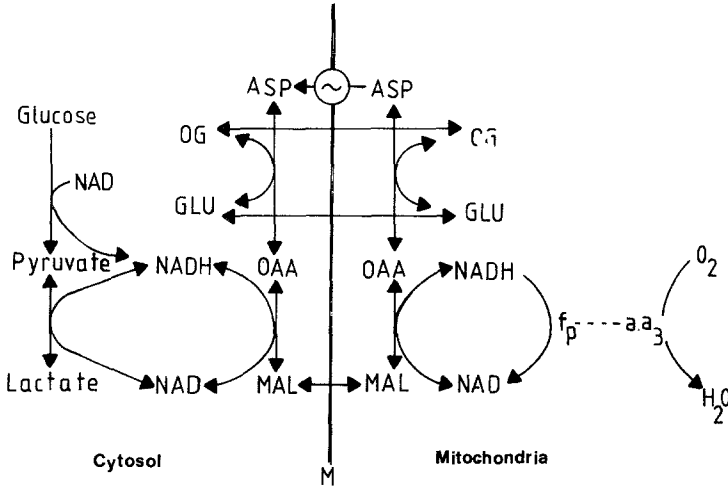
Fig. 1. The linking of the lactate/pyruvate equilibrium to the intramitochondrial redox state. The equilibrium between pyruvate and lactate at lactate dehydrogenase and between malate and oxaloacetate at malate dehydrogenase is linked through the NAD/NADH redox couple in the cytosol. The intra- and extramitochondrial aspartate aminotransferases and the malate, oxoglutarate, glutamate, and aspartate transporting systems in the mitochondrial membrane permit the metabolites of the intra- and extramitochondrial malate dehydrogenases to communicate. The intramitochondrial and extramitochondrial NAD/NADH couples are therefore in communication. However, the cytosolic couple remains much more oxidized because of electrogenic expulsion of aspartate from the mitochondrial compartment.

a fashion which is affected by the ratio $(ATP)/(ADP)(P_i)$ experienced in the cell (Sussman *et al.*, 1980; Erecinska and Wilson, 1982).

The redox state of the cytosolic NAD/NADH couple in cultured skin fibroblasts does not seem to be different from that observed in most mammalian tissues. However, the division of labor between glycolysis and the citric acid cycle/oxidative phosphorylation in terms of ATP production is different, with glycolysis accounting for 50% of ATP production so long as glucose is available. How does this come about? The most likely explanation is that in certain cell types, namely skin fibroblasts, platelets, lymphocytes, brain, and kidney medulla, a distinct form of phosphofructokinase (PFK-F) occurs which has no allosteric activators and is weakly inhibited by ATP and citrate (Meienhofer *et al.*, 1980). In cell types where this enzyme predominates, glycolysis is a very important factor in ATP generation, and lactate production occurs in the presence of adequate supplies of both respiratory substrate and oxygen. This unregulated F-type PFK is also present in significant amounts in tumor cells and fetal tissues where glycolysis is again a prominent mode of energy production (Davidson *et al.*, 1983). This mode of metabolism is also assisted by the presence

of a high titer of glycolytic enzymes and by the lack of mitochondria in these cells.

Estimation of the Redox State of the Cytosolic NAD/NADH Couple

Having outlined the mode of operation of energy metabolism in skin fibroblasts, let us examine some data from fibroblasts subjected to a simple experimental protocol (Table I). These cells are incubated in a buffer containing 1 mM glucose and at the end of this period the cells and buffer are analyzed for lactate and pyruvate. Control cells produce about 500 nmol of lactate and about 25 nmol of pyruvate giving an L/P ratio between 20 and 30 to one. When the pyruvate dehydrogenase is deficient, higher rates of lactate and pyruvate production are seen with an L/P ratio usually below the normal range. When there is a defect in the respiratory chain, located either in the NADH-coenzyme Q reductase or in cytochrome oxidase, the L/P ratio is elevated, with values ranging from 57 to 221. This is caused by a combination of an increase in lactate produced, sometimes to double the normal amount,

Table I.   Lactate and Pyruvate Production in Cultured Skin Fibroblasts[a]

	Lactate produced	Pyruvate produced	
	nmol/hr/mg protein		Lactate/pyruvate ratio
Controls			
1	591 ± 42(11)	28.5 ± 5.6(11)	26.9 ± 1.6(11)
2	537 ± 66(8)	21.1 ± 2.6(8)	24.1 ± 2.4(8)
3	459 ± 48(7)	24.5 ± 4.3(7)	21.5 ± 3.4(7)
4	465 ± 24(7)	21.7 ± 2.4(7)	23.4 ± 2.5(8)
PDH-deficient			
5	710 ± 58(8)	33.4 ± 4.7(6)	21.8 ± 3.8(6)
6	825 ± 95(4)	49.4 ± 15.3(4)	14.8 ± 1.9(4)
7	565 ± 73(3)	53.5 ± 4.5(3)	10.9 ± 2.1(3)
Cytochrome oxidase-deficient			
8	599 ± 66(4)	5.4 ± 1.9(4)	169 ± 67(4)
9	1089 ± 39(4)	5.1 ± 0.6(3)	221 ± 35(3)
10	910 ± 224(4)	6.7 ± 2.5(4)	189 ± 92(4)
11	760 ± 156(4)	13.9 ± 1.6(4)	57 ± 10(4)
NADH CoQ reductase-deficient			
12	965 ± 99(11)	9.7 ± 1.7(11)	98 ± 14(11)
13	921 ± 74(4)	8.7 ± 1.1(4)	104 ± 19(4)
14	1188 ± 103(7)	8.1 ± 1.6(7)	182 ± 45(7)
15	882 ± 76(4)	9.1 ± 1.8(4)	107 ± 21(7)

[a] Confluent skin fibroblast cultures were incubated with Krebs phosphate buffer for 1 hr. At this time the buffer was siphoned off and replaced by Krebs phosphate containing 1 mM glucose. After 1 hr perchloric acid was added to stop the reaction and the resulting extract assayed for lactate and pyruvate (Robinson et al., 1986).

and a reduction in pyruvate to one-third or one-fourth of the normal amount. Thus the inability to metabolize pyruvate through the pyruvate dehydrogenase complex because of PDH deficiency results in increased levels of pyruvate, which in being converted in part to lactate leads to a slightly more oxidized NADH/NAD couple in the cytosolic compartment and a lower L/P ratio. When the respiratory chain activity is compromised, resulting in increases in mitochondrial NADH/NAD and, by equilibrium, cytosolic NADH/NAD, this causes an increased L/P ratio through the lactate dehydrogenase equilibrium. Because of the slowed activity of the Kreb's cycle and respiratory chain, there is an increase in the total amount of lactate produced as the glycolytic pathway attempts to make up the deficit of ATP production.

Measurement of 1-^{14}C-Pyruvate Oxidation

Another simple test that gives an indication of a defect either in the PDH complex or the respiratory chain is the incubation of trysinized cells with 1-^{14}C-pyruvate with measurement of ^{14}CO$_2$ evolved (Robinson et al., 1980). In both types of defect the rate of 1-^{14}C-pyruvate oxidation to ^{14}CO$_2$ is decreased. In Fig. 2 the rates of ^{14}CO$_2$-production from 1-^{14}C-pyruvate have been determined with control cell lines and cell lines with either defects in the pyruvate dehydrogenase complex or defects in the respiratory chain. For most of the defective cell lines, ^{14}CO$_2$ production is statistically different from that in control cell lines. The reason why the defects do not have a more dramatic effect on the observed evolution of ^{14}CO$_2$ from 1-^{14}C-pyruvate almost certainly lies in the fact that neither the respiratory chain activity nor the activity of the pyruvate dehydrogenase complex are the rate-determining steps for pyruvate oxidation in trypsinized cultured skin fibroblasts.

Scope of the Defects Detectable in Fibroblasts

Localization of Defects in the Respiratory Chain

Ultimately once L/P ratios and pyruvate oxidation have been assessed in a cell culture, measurement of the suspected deficient enzyme itself must be performed. Micromethods for the measurement of pyruvate carboxylase (Atkin et al., 1979) and the pyruvate dehydrogenase complex (Hyland and Leonard, 1983) have been applied successfully to cultured skin fibroblasts. Localization of respiratory chain enzyme defects is more difficult because of the problem of making sufficient mitochondria from fibroblast cultures to perform assays.

We introduced a method of assessing skin fibroblast cultures with respiratory chain defects which could be performed with as little as 1 mg of
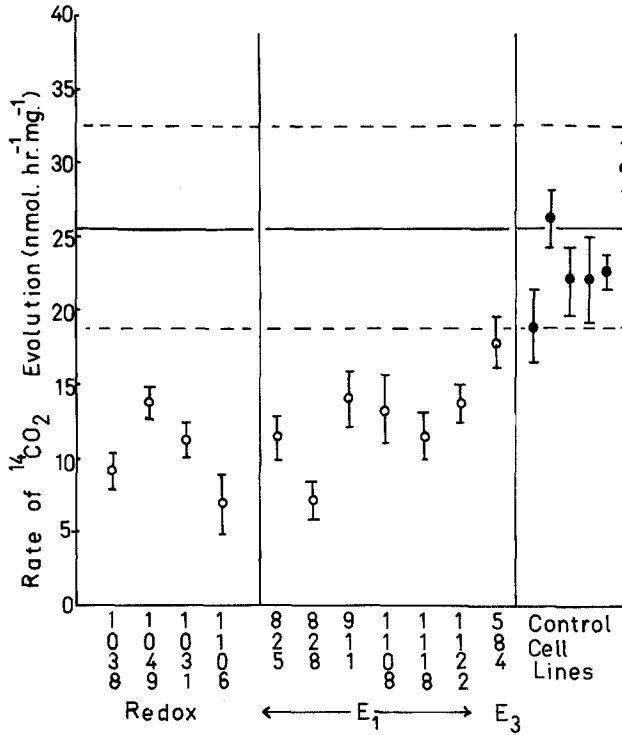
Fig. 2. Rate of $^{14}CO_2$ evolution from 1-^{14}C-pyruvate in trypsinized cultured skin fibroblasts. Rate of $^{14}CO_2$ evolution was measured as described in Robinson et al. (1980) in a number of different trypsinized fibroblast cultured cell lines. Cell lines were: 1038, 1049, 1106—NADH-CoQ reductase deficient; 1031—cytochrome oxidase deficient; 825, 828, 911, 1108, 1118, 1122—pyruvate dehydrogenase-E_1 decarboxylase deficient; 584—pyruvate-dehydrogenase-E_3 lipoamide dehydrogenase deficient. Six control cell strains.

fibroblast cell protein. In this method cells were first permeabilized with digitonin, then provided with ADP inorganic phosphate and a substrate for mitochondrial respiration. The amount of ATP produced in a unit time period could be compared to that produced by control cells and deductions made about the site of the defect (Robinson et al., 1985). These observations could be confirmed by polarographic measurement of oxygen consumption and by measurement of the activities of NADH-cytochrome reductase (rotenone sensitive), succinate-cytochrome c reductase, and cytochrome c oxidase in isolated mitochondria (Robinson et al., 1986).

The Pyruvate Dehydrogenase Complex

The pyruvate dehydrogenase complex is a macromolecular assembly of proteins which carries out the oxidative decarboxylation of pyruvate to form

Table II. Clinical Sequelae of Defects Diagnosable in Fibroblasts

PDH		Respiratory chain	
E$_1$	E$_3$	Complex I	Complex IV
Lacticacidemia	Lacticacidemia	Lacticacidemia	Lacticacidemia
Hypotonia	Hypotonia	Hypotonia	Hypotonia
Normal	Normal	Increased	Increased
L/P	L/P	L/P	L/P
—	Increased 2-oxoglutarate and branched chain amino acids	—	—
Psychomotor Retardation	Psychomotor Retardation	Psychomotor Retardation	Psychomotor Retardation
—	—	—	Nystagmus
—	—	—	Anorexia
Lesions in brain stem, basal ganglia, cerebral cortex	Lesions in brain stem, basal ganglia	Lesions in brain stem, basal ganglia *or* spongiform encephalopathy	Lesions in brain stem, basal ganglia
—	—	Cardiomyopathy	Cardiomyopathy (rarely)
50% have facial dysmorphism	—	—	—
Therapeutic response to low CHO diet	—	—	—
Four cases—agenesis of the corpus callosum			

acetyl coenzyme A. It consists of three major catalytic components, a thiamine pyrophosphate-dependent pyruvate decarboxylase (E$_1$), a transacetylase (E$_2$) which contains covalently bound lipoic, acid and dihydrolipoyl dehydrogenase E$_3$ which is strongly dependent on flavin adenine dinucleotide (Reed, 1981; Randle, 1983). In addition, there is a pyruvate dehydrogenase kinase, which inactivates the E$_1$ component (and thereby the whole complex) by phosphorylation of serine residues on the α subunits of the E$_1 \alpha_2 \beta_2$ tetrameric protein (Linn *et al.*, 1969). Activity is restored by the action of a Ca^{++}-dependent pyruvate dehydrogenase phosphatase which selectively removes the phosphate groups from the E$_1 \alpha$ serine residues.

The defects observed in the pyruvate dehydrogenase complex fall into two main groups, those with a defect in the first component, the E$_1$ decarboxylase, and those with a defect in the E$_3$ component, the dihydrolipoyl dehydrogenase. The most common of the defects is the E$_1$ deficiency, at least 50 cases of this having been identified worldwide. The majority of these cases have been localized to the E$_1$ component simply by means of assaying the isolated activities of the individual components of the complex (Robinson *et al.*, 1987a). In a few cases more detailed structural studies have been carried

out using either Western Blotting or ^{35}S-methionine labelling followed by immunoprecipitation. Ho *et al.* (1986) showed that a patient with severe pyruvate dehydrogenase deficiency had no detectable bands for either $E_1\alpha$ or β subunits on immunoblotting of fibroblast extracts. Wicking *et al.* (1986) showed that fibroblasts from a patient with severe PDH deficiency had no detectable band corresponding to $E_1\alpha$ by immunoblotting. In another less severe case there was changed mobility of the $E_1\alpha$ band when immunoblotted from two-dimensional polyacrylamide SDS electrophoresis/isoelectric focussing gels. McKay *et al.* (1986) showed that three patients with severe deficiency had altered mobility of the $E_1\alpha$ band on SDS polyacrylamide gel electrophoresis of the ^{35}S-methionine-labelled proteins.

The isolation of full length cDNA clones corresponding to the PDH-$E_1\alpha$ protein by Dahl *et al.* (1987) and ourselves (De Meirleir *et al.*, 1988) has permitted the examination of deficient cell lines at the level of mRNA by Northern Blotting techniques. Two distinct bands show up in normal cultured skin fibroblast mRNA, one species of the correct size for the precursor protein ($M_r = 45,000$) and another which is almost twice the size corresponding to the unprocessed transcript (Fig. 1). We have examined 12 cell lines from patients with E_1-PDH deficiency and all of them including those with abnormal bands on SDS/PAGE electrophoresis have a normal banding pattern on Northern Blotting. Dahl *et al.* (1987) report that their patient cell line with absent $E_1\alpha$ protein bands displays a reduced amount of the lower-molecular-weight mRNA with an increased amount of the unprocessed higher-molecular-weight mRNA. Thus the evidence points to the fact that some patients have a defect which resides in the $E_1\alpha$ subunit of the protein, but until we equally obtain information about the $E_1\beta$ subunit we cannot say how frequent the defects are in each of the subunits.

Lipoamide Dehydrogenase Deficiency

Deficiency of the third component of the pyruvate dehydrogenase complex is much less common than the E_1 group of deficiencies, there being only six well-documented cases in the literature. The fact that the E_3 component of the PDH complex is also the third component of the other α-ketoacid dehydrogenase complexes dictates that patients with this defect also have deficient 2-oxoglutarate dehydrogenase and branched-chain dehydrogenase complexes. The presence of this multiple defect leads to elevation of not only lactic acid in the body fluids of the patients but also elevations of 2-oxoglutarate, and the branched chain amino acids are often observed (Robinson *et al.*, 1978; Taylor *et al.*, 1978; Munnich *et al.*, 1982; Kuhara *et al.*, 1983). Urine samples display elevations of lactic, pyruvic, α-hydroxy-butyric, α-hydroxyisovaleric, α-ketoglutaric, and α-ketoisocaptroic acids (Robinson *et al.*, 1981; Matalon *et al.*, 1983).

Immunoextraction of lipoamide dehydrogenase from fibroblast proteins after ^{35}S-methionine labelling of proteins showed normal amounts of protein synthesized in three cases of deficiency and SDS/PAGE gels showed the relative molecular mass to be the same as in controls (Otulakowski et al., 1985). More recently, a full-length cDNA clone for human lipoamide dehydrogenase has been isolated in our laboratory (Otulakowski and Robinson, 1987).

Clinical Sequelae of Pyruvate Dehydrogenase and Respiratory Chain Defects

There were three groups that we could identify in the patients with pyruvate dehydrogenase (E_1) deficiency. The amount of residual enzyme activity in patients with this defect is quite variable. The presentation varies from overwhelming lactic acidosis at birth resulting in neonatal death to mild lacticacidemia with symptomatic episodes of ataxia occurring either episodically or associated with carbohydrate ingestion (Robinson et al., 1987a). Very low activity of the pyruvate dehydrogenase complex is often associated with severe lacticacidemia and death before 6 months of age. About one-third of patients with the defect die before two years of age, but most of those that survive suffer from psychomotor retardation. The majority of survivors go on to display varying degrees of structural brain damage that may become evident through brain scans carried out by computerized tomography or magnetic resonance imaging. The observed damage is seen in the brain stem, basal ganglia, and cerebral cortex in the form of cystic lesions which appear as a result of necrosis on discrete areas. All patients with the E_3 lipoamide dehydrogenase deficiency appear to develop these lesions, but only in the basal ganglia and brain stem. Lastly, there is a small group of patients with mild pyruvate dehydrogenase (E_1) deficiency who do not become mentally retarded but who suffer only from intermittent ataxic episodes. These episodes are often precipitated by carbohydrate ingestion and an increase in blood lactic acid. In fact, most patients with this defect benefit from a low carbohydrate/high fat diet. About half of the patients with PDH complex deficiency exhibit facial dysmorphism which is similar in appearance to the fetal alcohol syndrome. We have speculated that this similarity may indicate that fetal alcohol syndrome is caused by inhibition of the PDH complex by aceteldehyde derived from incomplete metabolism of alcohol.

The main biochemical indicator that differentiates respiratory chain defects from those of the PDH complex is the lactate-to-pyruvate ratio which is usually increased (Table II). Again there is some variability in severity but in general the complex I defects are evident sooner and have lactic levels which are elevated enough to cause problems with acidosis (Robinson et al., 1987c).

The patients with complex IV deficiency that can be diagnosed in fibroblast cultures generally have lactates which are elevated only two to three times normal and do not pose a problem early in life. They generally are poor feeders and are somewhat hypotonic, but they generally do not reach the appropriate milestones of sitting, crawling, walking, and talking until much later than they should. At this stage they are brought to medical attention. These patients very often regress in their abilities from two years of age and die between four and six years of age. During this period development of almost identical necrotic and cystic lesions to those seen in PDH complex deficiency occur (Robinson et al., 1987; Miyabayashi et al., 1985). In general these patients have between 17 and 28% of normal cytochrome oxidase activity in their cultured skin fibroblasts. We have diagnosed two patients as having a problem based on their having 40% of normal cytochrome oxidase activity and an increased lactate-to-pyruvate ratio. One of these cases, which had a clinical presentation and course as described for the other patients, appeared to have a kinetic defect in the complex which made it unusually sensitive to inhibition by oxidized cytochrome c (Glerum et al., 1987). The second case also had 40% of normal activity in fibroblasts but had only 17% in muscle (Chang et al., 1987). However, the symptoms in this case were more severe and death occurred early due to a severe cardiomyopathy.

These seems to be some cases of complex I deficiency that are almost identical to those seen in complex IV deficiency. They develop the same brain lesions and have an increased lactate-to-pyruvate ratio both in the patient and in the cultured fibroblasts.

Conclusion

Clearly, the research into the etiology of hereditary lacticacidemia has led to the appreciation that defects in the central oxidative pathways of human cells can and do occur. Further analysis of these defects is facilitated if the defect is reproducible in cell culture, and it will be the group of mitochondrial diseases which are expressed in skin fibroblasts which will be investigated most intensely and which will yield the most information from the original gene defect at the molecular level to the effect of that defect on somatic metabolism.

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