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ARTICLES

Diagnostic Value of Immunostaining in Cultured Skin Fibroblasts from Patients with Oxidative Phosphorylation Defects

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ABSTRACT: In the last decades, a large variety of oxidative phosphorylation (OXPHOS) defects have been reported, expressed as an increasing variety of clinical phenotypes. With the expanding number of genes and proteins involved, new screening techniques leading to more effective diagnostic routes are in ever-increasing demand. Cultured skin fibroblasts from a cohort of patients with various OXPHOS defects, previously recognized by enzyme activity studies and blue native PAGE, were investigated with an immunocytochemical technique. Cytospins of cultured fibroblasts were air dried, fixed, and stained with antibodies specifically directed against subunits of each OXPHOS complex. Control cells stained homogeneously and strongly. In fibroblasts from five out of seven patients with a severe deficiency of one of the OXPHOS complexes, a homogeneous reduction of cytoimmunoreactivity of the affected complex was observed. In five out of seven fibroblast strains harboring a mitochondrial tRNA mutation, a mosaic pattern of staining was observed for both complexes I and IV, reflecting the heteroplasmic nature of the defect. The proportion of deficient fibroblasts varied considerably between cell strains from different subjects. The method described offers a convenient and rapid approach to first-line screening of OXPHOS defects. In association with routine assays of enzyme activity, the technique is helpful in orienting molecular investigation further. (***Pediatr Res* 59: 2–6, 2006**)

Recent remarkable progress in understanding the genetics and molecular physiology of OXPHOS has revealed both its intrinsic structural complexity and the causal heterogeneity of mutations affecting mitochondrial function adversely. Moreover, besides the so-called nosological classic clinical entities mainly caused by mutations in the mitochondrial tRNA genes, genotype-phenotype correlation remains difficult in many mitochondrial disorders. The OXPHOS is run by a set of five multiprotein complexes, embedded in the lipid bilayer of the inner mitochondrial membrane: complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol:cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase). This structural system harbors at least 85 proteins, 13 of which are encoded by the small mitochondrial genome. Coordinated function of the complexes establishes a proton gradient between the mitochondrial matrix and the intermembrane space that, by the catalytic action of complex V, generates ATP.

Besides many mutations reported in the structural complexes themselves (1,2), mutations were discovered in nuclear genes encoding nonstructural proteins essential for assembly, importation, and folding of functional OXPHOS complexes. Examples of the former have been reported for complex III (3), IV (4), and V (5). Also, mutations in heat shock protein 60, a mitochondrial matrix chaperone, have been identified as the cause of fatal systemic mitochondrial disease (6).

As the phenotypic spectrum of OXPHOS defects rapidly expands and the molecular complexity steadily increases, the molecular diagnosis in the individual patient becomes laborious and expensive. There is a growing need for screening tests in the diagnostic laboratory, in particular methods with reliable discerning power between defects due to mutations either in mitochondrial or in nuclear genes (7). Specific antibodies raised against several subunit proteins in OXPHOS complexes have now become available. We here evaluate the

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Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; **MELAS,** mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; **MERRF,** myoclonic epilepsy and ragged-red fibers; **mtDNA,** mitochondrial deoxyribonucleic acid; **NARP,** neuropathy ataxia retinitis pigmentosa; **nuDNA,** nuclear deoxyribonucleic acid; **OXPHOS,** oxidative phosphorylation

value of using those antibodies in an immunocytochemical screening technique, for the identification of OXHPOS defects.

METHODS

Controls and patients. Cultured skin fibroblasts from 60 individuals were studied (14 patients, 46 controls). Skin biopsies were obtained after informed patient or parental consent and the study was approved by the University Hospital Review Board. Control fibroblasts were from patients with unrelated disorders: 10 patients had suspected collagen defects and 36 were patients from our pediatrics department that presented with various complaints of nonmitochondrial origin. To evaluate the usefulness of the diagnostic method described, patients with proven OXPHOS defects were included. Table 1 lists relevant clinical data on the 14 patients studied, representing various nosological phenotypes. Patients were subdivided according to deficiency of OXPHOS enzyme activities detected by spectrophotometric and BN-PAGE analysis and, where applicable, according to the results of mtDNA mutation analysis. The first group (patients 1–7) included patients with severe isolated complex deficiency. In two of them (patients 1 and 7), a mutation in nuDNA had been found (5,8). The second group comprised patients in whom the causal mtDNA mutation had been identified (patients 8–14).

Enzymatic studies. Spectrophotometric assays in skeletal muscle and liver had been performed for the measurement of complex I (NADH:ubiquinone oxidoreductase, rotenone sensitive) (9), complex II (succinate:ubiquinone oxidoreductase, malonate sensitive) (10), complex III (ubiquinone:cytochrome *c* oxidoreductase, antimycine sensitive) (11), complex IV (cytochrome *c* oxidase) (12), complex V (ATPsynthase, oligomycine sensitive) (10), and citrate synthase (13) enzyme activities. In cultured skin fibroblasts, only the activities of complex II, III, and IV were measured. In all tissue aliquots protein concentration was in the range of 2–4 mg/mL.

BN-PAGE. Native protein samples of skeletal muscle, liver, and cultured skin fibroblasts were subjected to BN-PAGE and to subsequent staining for catalytic activity of the OXPHOS complexes I, II, IV, and V as described (14).

Cytospins. Fibroblasts were cultured from skin biopsies according to standard procedures and harvested between the 5th and 10th passage at a confluence of approximately 80%. After discarding the culture medium, cells were washed with PBS. Subsequently, 1 mL of PBS was added to each T25 culture flask. Cells were detached with a rubber policeman and suspended by gentle agitation. Semi-thick cytospins were obtained by centrifugation (900 rpm, 5 min) in a Cytospin2 (Shandon, Pittsburgh, PA). Cytospins were air dried, fixed for 2 min with ice cold acetone, and stored at −80°.

Immunocytochemistry. Cytospins of fibroblasts were rehydrated in PBS for 5 min. For blocking of endogenous biotin, cells were incubated for 10 min with avidin and biotin (DAKO, Glostrup, Denmark) and then washed with PBS for 5 min. The cells were subsequently incubated for 30 min with 2.5% BSA in PBS. Dilutions of primary antibody were prepared in the same solution. Slides were incubated at room temperature for 1 h. Antibodies against one subunit in each complex were purchased from Molecular Probes, Eugene, OR (20 kD subunit for complex I (clone 20E9), IP subunit for complex II (clone 21A11), core2 subunit for complex III (clone 13G12), subunit I for complex IV (clone 1D6), and the α subunit for complex V (clone 7H10)). Antibody concentrations were 2 μg/mL, except for the antibody against the IP subunit of complex II (5 μg/mL). Immunoreactivity was amplified and visualized using the labeled streptavidin biotin2 system (DAKO) with DAB chromogen (DAKO). Nuclei were counterstained with hematoxylin solution (Sigma Chemical Co., St. Louis, MO) and mounted in Aquatex (Merck, Darmstadt, Germany). Positive staining was observed as a granular brown precipitate, which was scored as absent (0), weak (1), intermediate (2), or strong (3) by two researchers independently. Only the immunocytochemical scores of 0 and 1 were considered abnormal. In samples displaying mosaicism, mean proportion of negative fibroblasts was determined by averaging the count in three microscopic fields containing between 50 and 100 cells (AnalySIS software, Soft Imaging System, Lakewood, CO).

RESULTS

Fibroblasts from 46 control patients were strongly and homogeneously immunoreactive to all five OXPHOS complexes. Positive immunoreaction had a characteristic granular pattern, due to individual staining of mitochondria. Some cell cycle related variations in mitochondrial protein abundance were noticed, as the strongest staining was consistently observed in cells entering mitosis. The results of immunocytochemical staining in the cultured fibroblasts of the 14 patients are summarized in Table 2.

Five of the patients displayed an isolated defect in complex I activity (patients 1–5). In three of these patients (patients 2–4) a homogeneous reduction of complex I immunoreactivity was observed. Patients 1 and 5 displayed a normal staining pattern. Patient 6, in whom severe catalytic deficiency of complex II had been found, displayed a homogeneous reduction of complex II immunoreactivity (Fig. 1 *B*). In fibroblasts

Table 1. *Clinical, spectrophotometric, and BN-PAGE information on the cohort of patients*

			Skin fibroblasts		Other tissues		
Patient no.	Gender (age in years)	Clinical diagnosis	Spectrophotometric II–IV (I,V not measured)	BN-PAGE I–V	Spectrophotometric I–V	BN-PAGE I–V	Identified gene defect
			Patients with isolated OXPHOS complex defect				
1	F (<1)	Leigh syndrome	—	—	I↓ ↓ (SM)	I↓ ↓ (SM)	nuDNA complex I NDUFS4 (ref. 8)
2	M (<1)	Neonatal lactic acidosis	N	—	I↓ ↓ (SM, L)	—	
3	F (18)	Cardiomyopathy	N	I↓	I↓ ↓ (SM)	—	
4	F (14)	Cardiomyopathy	N	—	I↓ ↓ (SM)	I↓ ↓ (SM)	
5	F (4)	Encephalomyopathy	N	—	I↓ ↓ (SM)	—	
6	M (<1)	Infantile lactic acidosis	II↓ ↓	—	II↓ ↓ (SM, L)	II↓ ↓ (SM, L)	
7	F (<1)	Infantile lactic acidosis	—	V↓ ↓	N (SM, I + IV↓ (L)	V ↓ (SM, V ↓ ↓ (L)	nuDNA complex V ATP12 (ref. 5)
			Patients with mitochondrial DNA mutations				
8	F (36)	MERRF	N	—	—	I + IV ↓ ↓ (SM)	mtDNA $tRNA^{Lys}$ A8344G
9	F (36)	MERRF	—	I ↓ ↓	—	—	mtDNA $tRNA^{Lys}$ T8362G
10	M (<1)	MERRF	N	I + IV ↓ ↓	—	—	mtDNA $tRNA^{Lys}$ A8344G
11	F (29)	MERRF	—	I ↓	I + IV↓ ↓ (SM)	—	mtDNA $tRNA^{Lys}$ A8344G
12	F (51)	MELAS	—	N	N		mtDNA $tRNA^{Leu}$ A3243G
13	M (<1)	Nephroencephalomyopathy	—	—	I + IV↓ ↓ (SM)	I + IV ↓ (SM)	mtDNA $tRNA^{Asn}$ *
14	F (17)	Myopathy	N	N	I + IV ↓ ↓ (SM)	—	mtDNA $tRNA^{Asp}$ *

Enzyme activities not determined (—), normal (N), severely reduced (↓ ↓), moderately reduced (↓); tested in skeletal muscle (SM); liver (L). *Patients 13 and 14 carry a tRNA mutation not previously reported (submitted for publication).

Table 2. *Immunotyping of fibroblasts*

	Complex I (20 kd unit)	Complex II (IP subunit)	Complex III (core 2)	Complex IV (subunit I)	Complex V (subunit α)
1	2	3	2	2	2
2	1	2	2	2	2
3	1	2	2	3	2
4	1	2	2	2	2
5	3	3	3	3	3
6	2	1	2	2	2
7	2	3	2	3	0
8	M (19.3 ± 6.8%)	2	2	M (38.9 ± 15.8%)	3
9	M (66.5 ± 3.5%)	3	2	M (85.1 ± 3.1%)	2
10	M (84.2 ± 4.7%)	2	2	M (67.3 ± 4.2%)	2
11	M (84.5 ± 4.6%)	2	2	M (58.9 ± 2.9%)	2
12	M (25.5 ± 5.5%)	3	3	M (43.2 ± 11.4%)	3
13	M (29.7 ± 5.5%)	3	3	3	2
14	1	2	2	3	3

Results for immunoreactivity are indicated using conventional scoring of negative (0) to strong staining (3) and mosaicism (M), in which heterogeneous staining is indicated as mean percentages of deficient fibroblasts ± standard deviation. ND, not determined

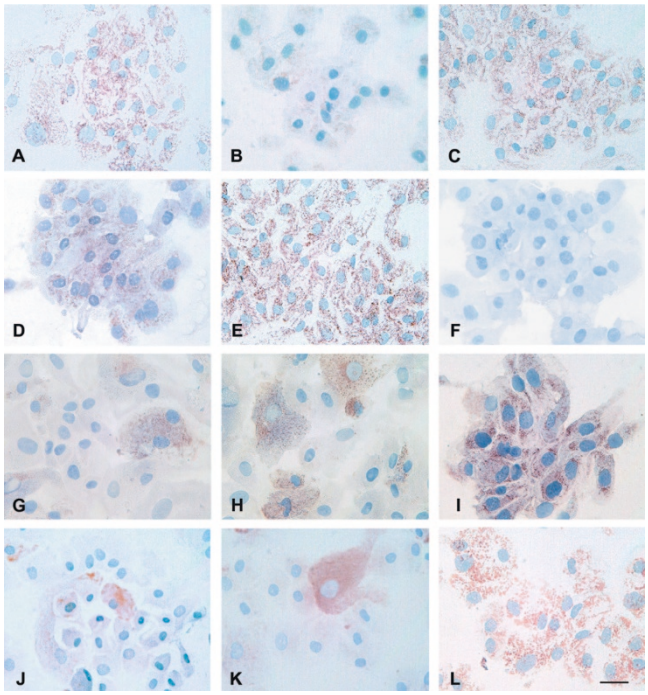

Figure 1. Immunocytochemical staining of fibroblasts for OXPHOS constituents (DAB, brown) with counterstained nuclei (hematoxylin, blue). *Patient 6*: (*A*) Normal staining for complex I subunit of 20 kD, (*B*) homogeneously reduced staining for complex II subunit IP, (*C*) normal staining for complex V subunit alpha. *Patient 7*: (*D*) Normal staining for complex I subunit of 20 kD, (*E*) normal staining for complex II subunit IP, (*F*) absence of immunostaining for complex V subunit alpha. *Patient 8*: (*G*) Mosaicism of positive and negative cells along side for complex I subunit of 20 kD, (*H*) mosaicism for complex IV subunit I, (*I*) normal staining for complex V subunit alpha. *Patient 10*: (*J*) Mosaicism for complex I subunit of 20 kD, (*K*) mosaicism for complex IV subunit I, (*L*) normal staining for complex V subunit alpha. Scale bar = 55 μm.

from the patient with severe deficiency of complex V due to a nuclear ATP12 gene defect (patient 7), immunoreactivity to complex V was completely abolished (Fig. 1*F*).

Patients 8–11 with proved MERRF and patient 12 with proven classical MELAS displayed combined mosaicism for complex I and complex IV (Fig. 1, *G*, *H*, *J*, and *K*). In the majority of fibroblast strains of this group of patients, negative cells were observed alongside normally staining fibroblasts. In the strain derived from patient 12, however, mosaic staining presented as high-density staining in some and little staining in other fibroblasts. Remarkably different was the immunostaining result in fibroblasts from patients 13 and 14, carrying nonclassical tRNA mutations. Patient 13 displayed mosaicism for complex I only, while patient 14 had homogeneous reduction of complex I immunoreactivity.

DISCUSSION

The rapidly expanding knowledge of mitochondrial biology as well as the increasing number of multisystemic disorders with mitochondrial causation and pathogenesis generates the need for developing reliable and easily accessible means of diagnosis. Any new approach needs the scrutiny of comparison with the successes and flaws recorded for methods currently in use. Immunocytochemical evaluation in cultured fibroblasts could be an elegant alternative technique, but, until now, this approach had not been evaluated thoroughly for its diagnostic potential.

The results presented show that immunocytochemical staining in fibroblasts is useful in predicting the underlying defect. In five of the seven patients with severe isolated complex deficiency, immunostaining for the corresponding complex was significantly decreased. The immunostaining in fibroblasts was consistently abnormal in all patients with a mtDNA mutation, and in most of them a mosaic staining pattern for both complex I and complex IV was obtained. Our findings confirm an earlier observation of intercellular mosaicism in cultured skin fibroblasts from five MELAS patients immunostained for subunit I and VIc of complex IV (15). Possible explanations for the normal staining in two patients with isolated complex I deficiency include i) the specific defect at hand is not expressed in skin fibroblasts, or ii) generation of the mature complex is blocked at the level of assembly and subcomplexes remain immunoreactive, or iii) a stable holocomplex has been composed but is not catalytically active.

Catalytic deficiency of OXPHOS complexes is, however, most often associated with a decreased amount of assembled

complex (5,16–20). The diagnostic value of the immunostaining techniques relies on this fact, as has been amply demonstrated by BN-PAGE followed by immunoblotting (16). mtDNA mutations affecting intramitochondrial protein synthesis represent one of the known causes of a dramatic reduction of assembled complexes. Most severely affected are the complexes composed of multiple mtDNA encoded subunits, as are complex I, complex IV, and, to a lesser extent, complex III. Such defects in mitochondrial complexes may be the results of a mtDNA deletion, or mtDNA depletion, or point mutations in tRNA or rRNA genes.

Known features of mtDNA mutations include heteroplasmy and the threshold phenomenon. Heteroplasmic cells have a mixture of mitochondria carrying normal mtDNA and mutant mtDNA. Only when the mutant mtDNA copy number exceeds the threshold 80–85%, synthesis of the encoded protein will be adversely affected (21). In skeletal muscle from patients with heteroplasmic mtDNA mutations, mosaic immunostaining for complex IV has been reported (22). It is shown here that a mosaic pattern can also be demonstrated among cultured skin fibroblasts. Hence, the detection of such a heterogeneous staining pattern in some of the cultured fibroblasts is a strong indication for a mtDNA defect. A homogeneous decrease of the immunoreactivity of one complex is more suggestive for a nuclear defect in a gene encoding a structural subunit, or a protein involved in assembly or import of the complex.

The method described differs from the earlier experimental protocols (15,23) and offers two major advantages for clinical practice. Firstly, the immunocytochemical techniques used previously were based on fluorescent detection requiring expensive and specialized apparatus. The protocol proposed here can be executed in any laboratory with standard equipment as it only depends on light microscopic evaluation. Secondly, experiments in previous studies were performed in fibroblasts cultured on chamber slides. For the purpose of immunotyping, fibroblasts can easily be prepared by centrifugation onto microscopic slides, a routine procedure used in all cytopathology labs. Although this procedure disrupts cell morphology, it has no effect on the antigenicity of OXPHOS complexes and does not impair sample interpretation. This way, cost and time of cultivation can be reduced considerably.

Skeletal muscle is the tissue preferentially examined for making the formal diagnosis of an OXPHOS defect. However, in some instances the investigation in cultured skin fibroblasts is very useful. Firstly, in some patients the OXPHOS defect is not expressed in skeletal muscle (24). In this case, a skin sample can be taken simultaneously with the skeletal muscle biopsy and used for cultured skin fibroblasts. Secondly, in very young children, a skeletal muscle biopsy is a much more invasive first-line diagnostic procedure, and a skin biopsy is a good alternative. Thirdly, in situations where either a skeletal muscle specimen or the patient is no longer available, investigation of OXPHOS defects in cultured fibroblast may still yield a precise diagnosis, important for genetic counselling of the family. Finally, mutant fibroblast strains are essential for complementation studies with rho-zero cells to find out whether the defect is located in the mitochondrial genome or in the nuclear genome (25).

Spectrophotometric analysis in cultured skin fibroblasts allows detection of isolated OXPHOS complex deficiencies of complex II, III, and IV. The enzyme assays for complex I and complex V, however, are less sensitive and, therefore, less reliable. Immunostaining for complex I and complex V in fibroblasts represents an alternative approach. Spectrophotometric analysis in fibroblasts is also less suitable for detection of more subtle decreases of catalytic activity as seen in patients with a limited copy number of mutated mtDNA. Using the immunocytological method presented here, the fibroblasts are evaluated individually allowing detection of even a small number of deficient cells.

We conclude that the immunocytochemical method described here may aid the diagnosis in mitochondrial disease considerably. The method does not hold the potential to substitute all diagnostic methods currently available for objective confirmation of OXPHOS deficiencies, but in any routine laboratory setting it is a valuable first-line screening test for patients suspected of mitochondrial metabolic disease. In laboratories specialized in this area, the method may be added to the battery of diagnostic tools comprising the spectrophotometric measurements of OXPHOS enzymatic activity, the BN-PAGE method, and DNA analysis.

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