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Characterization of cytochrome-*c* oxidase mutants in human fibroblasts

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Skin fibroblasts were selected as having cytochrome-*c* oxidase deficiency by activity mesurements in whole cells. Each cell line was cultured in sufficient amount to isolate mitochondria for biochemical characterization. Cytochrome-*c* oxidase was then examined by activity measurements, by heme determination and by polypeptide analysis using antibodies specific to the enzyme subunits. The cytochrome-*c* oxidase activity in the different cell lines ranged from 9% to 54% of that of normal fibroblasts. Heme determinations and polypeptide analysis established that the lowered cytochrome-*c* oxidase activity was due to reduced amounts of the complex in the mitochondrial inner membrane. In all cases, there was defective assembly of the enzyme, with the amounts of mitochondrially coded and nuclear coded subunits being reduced proportionally. These studies show that fibroblasts can be used for prenatal diagnosis of mitochondrial diseases and are a useful system in which to study mitochondrial biogenesis.

Cytochrome *c* oxidase; Heme; Mitochondria; Fibroblast

1. INTRODUCTION

Cytochrome-*c* oxidase, the terminal component of the respiratory chain, catalyzes the four electron reduction of molecular oxygen, coupling this to proton pumping across the mitochondrial inner membrane [1,2]. The mammalian enzyme has been found to consist of 13 different polypeptides [3,4], three of which are coded for by mtDNA [5], the remainder being encoded by nuclear DNA. It is the mitochondrially coded subunits which provide the catalytic core of cytochrome-*c* oxidase, as apoproteins for the redox centers and including the redox linked proton pump [1,2]. The nuclear coded subunits of the enzyme appear to have a regulatory function [6,7].

Heme cytochrome-*c* oxidase deficiency has been described in a number of patients [8-10] and we have shown that in certain cases the defect is demonstrable in cultured skin fibroblasts [11-13].

Here we describe a study of 13 different fibroblast cell lines, all from patients with lacticacidemia associated with either Leigh syndrome, Kearns-Sayre syndrome or other generalized mitochondrial myopathies. These were chosen because they showed cytochrome oxidase deficiency by activity measurements of whole cells. Mitochondria have been isolated from these cell lines and respiratory chain defects studied biochemically in fibroblasts for one of the first times (see also [10] published after completion of our work).

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2. MATERIALS AND METHODS

Cell lines used in this study were obtained by skin biopsy from patients, after informed consent. Fibroblasts were cultured in Eagle's minimum essential medium and 10% fetal calf serum, supplemented with glucose to a concentration of 10.5 mM. Mitochondria were isolated from confluent cultures of these cells by the method of Millis and Pious [14], omitting the use of protease. Cytochrome-*c* oxidase activity was assayed

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in sonicated mitochondria by the method of DiMauro et al. [15] and in sonicated whole cell preparations by the method of Glerum et al. [16].

Immunoblotting was carried out using 100 μg of fibroblast mitochondrial protein. Samples were separated on 6 M urea/16% SDS polyacrylamide gels (modified from Kadenbach et al. [17]) and electroblotted onto nitrocellulose by the method of Towbin et al. [18], with the addition of 0.1% SDS to the electrode buffer. The electroblotted nitrocellulose sheets were then blocked with 3% gelatin-TBS (20 mM Tris, 0.5 M NaCl, pH 7.5) for 1 h, followed by an overnight incubation in primary antibody. Incubation in secondary antibody was for 3 h, using affinity-purified goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad). The subunit specific antibodies used were to Mt_{II} and C_{IV} (nomenclature of Takamiya et al. [19]), and to the core proteins of complex III. The antibodies were raised to the purified subunits of the bovine heart enzyme.

Heme concentrations were determined from oxidized minus dithionite reduced spectra, in a Beckman DU-7 spectrophotometer. Mitochondrial preparations were used at a concentration of at least 3 mg/ml of protein, suspended in a 100 mM potassium phosphate buffer, pH 7.4, containing 1% Triton X-100. The concentrations were calculated using the extinction coefficients of Bookelman et al. [20] and protein was determined by the method of Lowry et al. [21].

3. RESULTS

3.1. *Electron transfer activities in cultured skin fibroblasts*

Control fibroblasts (taken from subjects without identified metabolic disturbances) proved to have lower electron transfer activities than found in other tissues. For example, the rotenone-sensitive NADH-cytochrome *c* reductase activity was 53 $nmol \cdot min^{-1} \cdot mg^{-1}$ and cytochrome-*c* oxidase activity 40 $nmol \cdot min^{-1} \cdot mg^{-1}$, compared with values of 130 $nmol \cdot min^{-1} \cdot mg^{-1}$ and 900 $nmol \cdot min^{-1} \cdot mg^{-1}$, respectively, for these two activities in human skeletal muscle [22]. This low content of cytochrome-*c* oxidase in fibroblasts was confirmed by determination of heme *a*. The heme *a* content of control fibroblasts was 21 pmol/mg mitochondrial protein, compared with values of around 500 pmol/mg in human skeletal muscle [22]. The ratio of cytochromes *b*, c_1 and aa_3 in fibroblasts calculated from heme determinations was 2.5:2.5:1 (cf. 1:2:2 in human skeletal muscle).

3.2. *Studies of mutant fibroblasts*

Cultured skin fibroblasts from patients with lacticacidemia were screened initially for respiratory chain defects by measuring lactate to pyruvate ratios as an indicator of intracellular redox state. Respiratory chain defects were then confirmed by activity measurements on cell cultures.

Eleven patient cell lines with normal NADH and succinate cytochrome *c* reductase activities but with decreased cytochrome-*c* oxidase activity as measured in whole cells were chosen for further study. These are listed in table 1. Two other cell lines were examined because they displayed consistently decreased activity of cytochrome-*c* oxidase, though their lactate to pyruvate ratios were only slightly above normal. Activities of cytochrome-*c* oxidase varied from 9 to 56% of the activity determined in control cell lines. Electron microscopic examination of the deficient cells revealed no abnormality of mitochondrial ultrastructure. Further analysis was carried out using mitochondria isolated from these skin fibroblasts.

3.3. *Heme determinations*

Visible spectra in the α-band region and in the Soret region for two of the cell lines deficient in cytochrome-*c* oxidase activity are shown in fig.1, along with the spectrum of a control fibroblast cell line. The amount of cytochrome aa_3 in relation to cytochromes *b* and *c* is significantly smaller in both, and is approximately proportional to the amount of cytochrome-*c* oxidase activity measured for these two cell lines. Thus in cell line 2417, there was no detectable aa_3 either by absorbance in the

Table 1

Cell lines used in this study

Cell line	Activity (%)
Control 1685	100
Partial defect	
1630	56
1947	50
2130	41
2233	45
2440	46
Severe defect	
890	24
1489	27
1909	19
2161	17
2246	25
2288	30
2417	9
2434	12

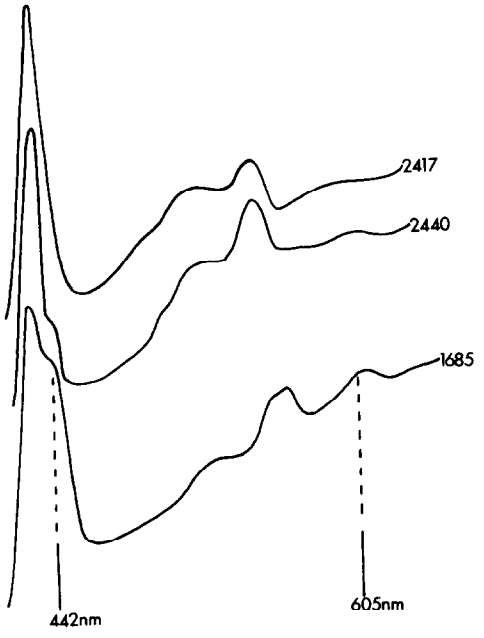

Fig.1. Reduced minus oxidized optical difference spectra of mitochondrial samples from cultured skin fibroblasts. Cell line 1685 is a control cell line. Cell lines 2417 and 2440 are from patients with Leigh's syndrome. Samples were reduced with sodium dithionite.

α-band region or in the Soret at 442 nm. This cell line shows only 9% normal cytochrome-*c* oxidase activity. Cell line 2440 shows a small peak for aa_3 at 605 nm and a shoulder at 442 nm in the Soret region, consistent with a small amount of cytochrome-*c* oxidase being present. This cell line shows 40% of normal cytochrome-*c* oxidase activity.

Table 2 summarizes the cytochrome-*c* oxidase activities and heme determinations for several different cell lines.

3.4. *Polypeptide analysis*

A typical immunoblotting experiment in which cell lines were tested for the presence of cytochrome-*c* oxidase subunits is shown in fig.2. Antibodies against subunits Mt_{II} and C_{IV} [19] were used to monitor the relative concentrations of the mitochondrially and nuclear coded subunits, respectively. As an internal control, antibodies against the core proteins of complex III were included. Each lane contains 100 μg of mitochondrial protein from a different cell line. It can be seen that the labelling by core protein antibody is, relatively, the same in each lane, confirming the activity measurements in showing that the various cell lines have normal amounts of complex III. The amounts of both subunits Mt_{II} and C_{IV} were diminished in the cytochrome-*c* oxidase deficient cell lines, roughly in proportion to the amount of enzyme indicated to be present from activity and heme determinations. Thus cell line 1630 has approximately half the amount of subunits Mt_{II} and C_{IV} and 50% activity, while cell line 2161, with only 20% normal activity has barely detectable amounts of the two cytochrome-*c* oxidase subunits. Likewise, lines 1489, 1947, 2130, 2233, 2288 and 2440 have decreased amounts of protein and 30–50% activity of cytochrome-*c* oxidase, whereas lines 890, 1909, 2246, 2417 and 2434 have very little if any detectable protein and cytochrome-*c* oxidase activities ranging from 25% down to 9%. It is interesting to note that antibody to subunit Mt_{II}

Table 2

Cytochrome-*c* oxidase activity and heme content of fibroblast cell lines

Cell line	Activity (nmol/min per mg mitochondrial protein)	Heme content (pmol/mg mitochondrial protein)			
		b	c_1	aa_3	bc_1:aa_3
Control	40.8 ± 4.9 (7)[a]	57	54	21	5:1
	4.3 ± 0.5 (5)[b]				
Def. 1630	23.6 ± 4.1 (6)[a]	77	53	16	8:1
2417	0.4 ± 0.2 (5)[b]	89	91	undetectable	-
2440	2.1 ± 0.3 (5)[b]	76	57	11	12.1
1909	7.7 ± 1.2 (9)[a]	25	21	undetectable	-

[a] Determined in mitochondria
[b] Determined in whole cell extracts

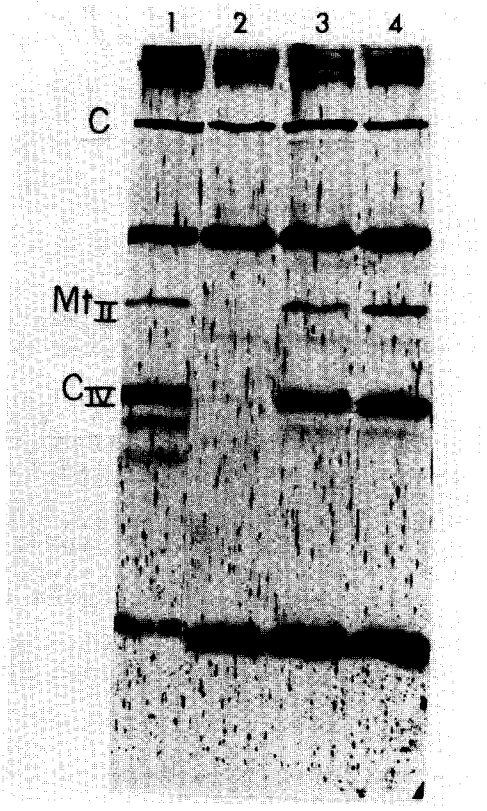

Fig.2. Immunoblots of mitochondrial samples from cultured skin fibroblasts. The cell lines being examined are (lanes): (1) 2233; (2) 2161; (3) 1630; (4) control cell line 1685. Band C is for the core proteins of complex III. Mt_{II} and C_{IV} are subunits of cytochrome-*c* oxidase.

reacts nonspecifically in fibroblasts with proteins of $M_r \simeq 30\,000$ and 11 000, respectively. This cross-reactivity has not been seen in immunological studies of other human tissues [22].

4. DISCUSSION

Fibroblasts constitutively have few mitochondria and are cultured in vitro, using glycolysis as the major energy-generating pathway. The spectral data presented here show that the heme concentrations in fibroblast mitochondria are low compared with most other mammalian tissue types and that the amount of cytochrome-*c* oxidase relative to complex III is also lower, i.e. 1:5 compared with 1:2 in human skeletal muscle [22] and 2:1 in heart [23].

Our data demonstrate that fibroblasts from a number of patients with lacticacidemia have a very low cytochrome-*c* oxidase content by activity, by the low amount of heme aa_3 present and by a reduced amount of titratable subunits in the immunoblotting experiments. In all of the cell lines reported here, the respiratory chain defect is localized to complex IV or cytochrome-*c* oxidase, and the amount of complex III (based on activities, heme determinations and immunoblotting with core protein antibody) as well as complexes I and II appears to be normal. Complexes I and III, like cytochrome-*c* oxidase, have both mitochondrially synthesized and nuclear coded subunits [23–25].

It has been established that there is a sequential assembly of cytochrome-*c* oxidase in rat liver mitochondria [26], and that a nucelar coded subunit is integral to this process. It has been shown by Wright et al. [27] that for the yeast enzyme, a deletion of the gene for subunit VIIa (corresponding to mammalian C_{IX}) results in the loss of a functional cytochrome-*c* oxidase holoenzyme, and mutant cells lacking subunit VIIa also lack many other cytochrome-*c* oxidase subunits. It has also been proposed that mitochondria possess a groEL-type protein responsible for refolding of newly imported proteins [28] which could also be important in the assembly of cytochrome oxidase.

Thus, the possibility exists that in the cell lines with reduced titratable cytochrome-*c* oxidase that there could be a mutation present which either (i) involves a gene encoding a mitochondrially synthesized subunit of complex IV, (ii) involves a gene encoding a subunit of complex IV synthesized in the cytoplasm or (iii) involves a gene encoding a structural or regulatory protein which is essential for the assembly of complex IV. The fact that the mode of inheritance for all these cases appears to be autosomal recessive makes the first possibility unlikely, but the exact site of the primary mutation is as yet obscure.

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