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Defects of mitochondrial respiratory chain in multiple symmetric lipomatosis

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Abstract **Using lymphocytes from nine unrelated patients with multiple symmetric lipomatosis we investigated a possible defect in the mitochondrial respiratory chain as the biochemical cause for the disease. A significant decrease in oxygen consumption of intact lymphocytes as well as a decreased activity of the individual components of the respiratory chain were detected. These findings are consistent with the recently described deletions and point mutations of mitochondrial DNA in patients suffering from this disease.**

Key words Multiple symmetric lipomatosis · Benign symmetric lipomatosis · Mitochondria · Respiratory chain

Introduction

Multiple symmetric lipomatosis (MSL) is a rare disorder characterized by symmetrical fat masses predominantly around the neck and shoulders [1, 2]. The ultrastructure of the adipose cells of the lipomas and the distribution of the fat masses has led to the suggestion that the fat cells might originate from brown fat which is present in human newborns at these locations and can persist into adulthood to different extents [3, 4]. In order to fulfil the main task of this tissue the specialized mitochondria of brown adipocytes produce heat rather than ATP [5]. Due to the importance of mitochondria for the tissue, a defect in the mitochondrial respiratory chain could be the elusive biochemical defect in MSL patients. All three enzymes of the mitochondrial respiratory chain are of dual genetic origin. While most of the subunits are encoded in nuclear DNA each enzyme harbours at least one essential subunit that is encoded on the separate mitochondrial genome. In this respect, it is interesting that deletions of mtDNA have been found in some patients suffering from MSL [6, 7]. These deletions most probably include essential rRNA and tRNA genes of the mtDNA and thus will affect the translation of all mitochondrially encoded subunits, thereby lowering the activity of all three respiratory complexes.

To avoid the critical and troublesome preparation of mitochondria, we determined the activities of the individual components of the respiratory chain polarographically in intact and digitonin-permeabilized white blood cells of nine unrelated MSL patients [8, 9].

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Materials and methods

Blood samples

A 100-ml blood sample withdrawn from each patient after obtaining informed consent was stabilized with 2% EDTA, diluted with 50 ml buffer (138 m*M* NaCl, 2 m*M* KCl, 8 m*M* Na_2HPO_4, pH 7.4) and layered over 4 × 12 ml Ficoll (Biochrom KG Seromed). After centrifugation (20 min, 840 *g*) lymphocytes were collected as a white layer on top of the Ficoll solution. Cells were washed in PBS twice and resuspended in medium (20 m*M* HEPES, pH 7.1, 250 m*M* sucrose, 10 m*M* $MgCl_2$). Cells that were not measured directly were incubated in RPMI medium (Gibco) supplemented with 5% fetal calf serum (Gibco) for a maximum of 1.5 h. Control samples were provided as buffy coats from the local blood bank and treated in the same way.

Polarography

Approximately 2×10^7 white blood cells were resuspended in respiration medium (20 m*M* HEPES, pH 7.1, 2 m*M* Na_2HPO_4, 250 m*M* sucrose, 10 m*M* $MgCl_2$ and 1.0 m*M* ADP) and introduced into the chamber of a Clark electrode. Two small aliquots were removed to determine the exact number of cells in the chamber. Substrates and inhibitors were added with Hamilton syringes from 100 × stock solutions (neutralized with NaOH when necessary). After recording the initial slope corresponding to the respiration rate on internal substrate, the cells were permeabilized with 300–400 µg digitonin. Complete permeabilization was confirmed by Trypan blue staining. Substrates and inhibitors were added and the corresponding slopes were recorded. Final concentrations were: malic acid, 5 m*M*; glutamic acid, 5 m*M*; succinate, 5 m*M*; glycerol-3-phosphate (G-3-P), 10 m*M*; ascorbic acid, 1 m*M*; *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 0.2 m*M*; rotenone 400 n*M*; antimycin A, 50 n*M*; KCN, 1 m*M* [9]. Two to four measurements were performed for each sample and the averaged data were analysed.

DNA analysis

Platelets were harvested from the blood samples of the patients and DNA was extracted using the Genomic tips according to the instructions of the supplier (Qiagen).

For the detection of the MELAS mutation the corresponding part of the mtDNA was amplified by PCR using a primer corresponding to position 3007–3023 of the heavy strand and position 4531–4512 of the light strand. With the mutation present the fragment should be cleaved with the restriction enzyme Apa I at position 4243. An internal Apa I site at position 4430 was used to check for incomplete digestion.

With primers corresponding to heavy strand position 8286–8305 and light strand position 8611–8590 a fragment including the site of the MERFF (nt 8344) mutation was amplified and compared with the corresponding fragment from a patient with the MERFF mutation by temperature gradient gel electrophoresis [10].

Deletions were detected with primers corresponding to heavy strand position 7293–7316 and light strand positions 13928–13905 and were used to amplify a 6.6-kb fragment encompassing the region of the common deletion (Expand Template PCR System, Boehringer Mannheim).

Results and discussion

The nine patients had a histological and clinical diagnosis of MSL. The data of the patients are summarized in Table 1. Control blood samples from healthy donors were obtained from the local blood bank. Lymphocytes were isolated by standard Ficoll gradient centrifugation and oxygen consumption of the intact cells was measured using a Clark electrode. Afterwards the individual components of the respiratory chain were analysed in digitonin-permeabilized cells. Figure 1 shows the individual data points as well as the average and the standard deviation of the measured activities of patient and control samples. The oxygen consumption of the intact cells was reduced by 31% compared with that of the control subjects. The activities for the three enzymes of the mitochondrial respiratory chain, Complex I (NADH, ubiquinone reductase), complex III (ubiquinol cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase), were reduced in the cells from the MSL patients by 36%, 27% and 23%, respectively. The differences between the means of control and patient lymphocytes oxygen consumption and complex I activity was greater than would be expected by chance (nonparametric Mann-Whitney *U*-test). However, the data for complex III and complex IV were not significantly different.

The results could have been biased because of the age-dependent decrease in oxidative phosphorylation. The median age of our patients was 52 years (SD ± 7, range 42–62 years) while that of the controls was 38 years (SD ± 14, range 18–62 years). According to an investigation by Trounce et al. this age difference should be associated with a less than 10% decrease in mitochondrial activity [11]. In addition, we were unable to detect age-related differences in the activities of our control group (data not shown). Thus, age alone cannot explain the differences we found.

Using blood cells, the extent of the defect in the lipoma of the patients might be actually underestimated. Due to

Table 1 Clinical features of nine patients with benign symmetric lipomatosis

No.	Age (years)	Sex	Age at onset (years)	Duration (years)	Underlying diseases	Alcohol abuse	Medication	Neurological symptoms
1	58	F	56	2	Alcohol toxicity-induced cirrhosis	Excessive	Silymarine, spironolactone, ornithine aspartate, metoclopramide	Depression
2	47	M	37	19	Hyperthyroidism	None	Levothyroxine	None
3	56	M	52	4	Struma diffusa, hypertension, bronchitis, tachycardia	None	Verapamil, digoxin, acetylsalicylic acid, metoprolol tartrate	None
4	49	F	39	10	Diabetes, hypertension, hyperlipidaemia	None	Verapamil, isosorbide dinitrate, metoprolol tartrate, bezafibrate, molsidomine	Polyneuropathy
5	42	M	39	3	Struma nodosa	None	None	None
6	62	F	58	4	Hyperuricaemia, cirrhosis	Denied	Propranolol, spironolactone, allopurinol, sodium fluorophosphate, calcium gluconate	None
7	57	F	54	3	Hyperthyroidism	None	Levothyroxine	None
8	50	M	44	6	Diabetes, hyperlipidaemia	Excessive	Glibenclamide	Polyneuropathy
9	46	F	28	18	Hypertension, mastodynia, hysterectomy	Denied	Amiloride, hydrochlorothiazide	None

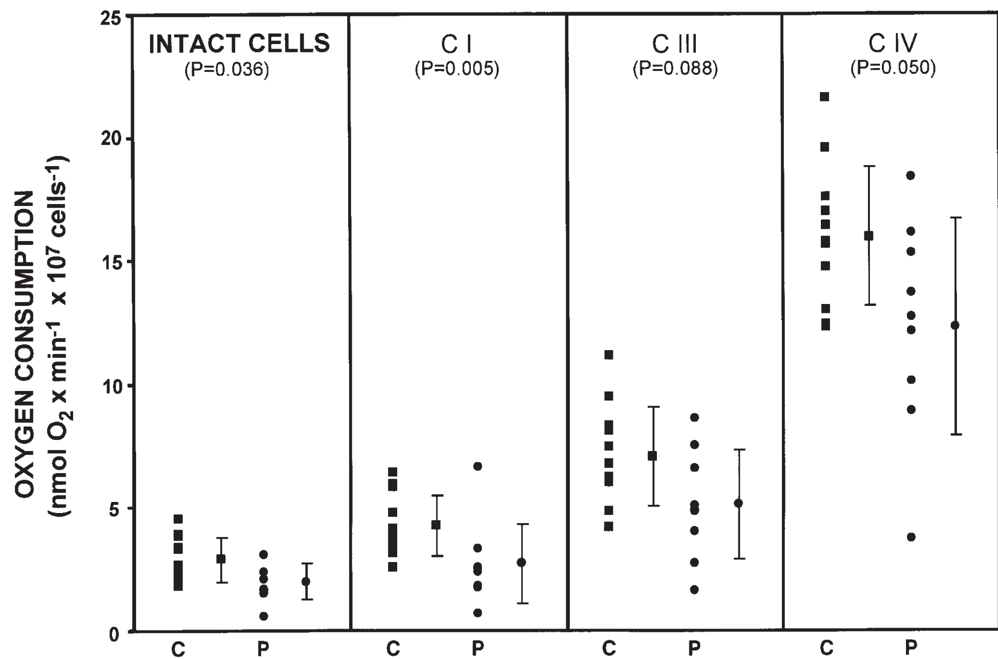

Fig. 1 Oxygen consumption of intact and permeabilized cells with malate/glutamate (CI), succinate/glycerol 3 phosphate (CIII) and TMPD/ascorbate (CIV) (*C* control cells, *P* patient cells). In addition the mean and standard deviation of the control and patient cells are shown. In brackets the significance for the difference between the means of the control and patient cells is given (Mann-Whitney *U*-test)

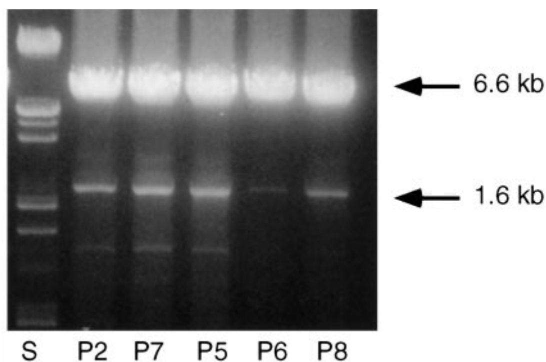

Fig. 2 PCR products from the amplification of mtDNA between nt 7293–13928 separated on agarose gel (*S* standard sample, *P* patient samples)

the high copy number of mtDNA a mixture of mutated and unmutated copies is frequently present in cells. The degree of mutated molecules varies between tissues and is frequently lower in blood cells. This has been demonstrated in a recently described MSL patient with the mitochondrial $tRNA_{lys}$ mutation associated with the myoclonus epilepsy and ragged red fibres (MERRF) syndrome [12]. The percentage of mutated mtDNA molecules in the blood cells of the patient was reduced compared to muscle and lipoma. Such segregation phenomena will increase the variance between individuals and might contribute to the fact that the activities for the lymphocutes of most patients were at the lower end, but still within the range of the activities of the control cells.

An association of the MERFF mutation with lipomas has been established in several other patients [13, 14]. Furthermore, deletions in mtDNA have been detected in some patients [6, 7]. However, in the majority of patients no distinct mitochondrial mutation has yet been observed. Likewise, when we investigated the mtDNA of five patients who volunteered another blood sample, we were not able to detect the MERFF mutation or the other well known mitochondrial $tRNA_{leu}$ (MELAS [15]) mutation (data not shown). Also the search for deletions in the most delicate region of the mtDNA (nt 8000–14 000) revealed only an insignificant percentage of deletions (Fig. 2). Thus, at present there is no specific mutation or deletion of mtDNA associated with this disease.

Mitochondrial deletions will most often include mitochondrial tRNA genes that are scattered throughout the mtDNA. Therefore, like tRNA mutations they will affect the entire mitochondrial translation. Such mutations should result in defects of all enzymes of the respiratory chain similar to what we found in the present investigation, rather than in isolated effects on cytochrome *c* oxidase reported in the literature [6, 7]. Likewise, in the majority of our patients all enzymes were affected. This might point to mitochondrial tRNA or rRNA genes as hot spots for mutations and deletions.

The preferential effect of mitochondrial mutations on the adipose tissue is presently unexplained. β-Oxidation and lipolysis in these cells might be reduced due to the limited reoxidation of the NADH by the mitochondrial respiratory chain. The accumulation of additional lipid might serve as a signal for the cell to divide and gain a growth advantage or survive the recession of the brown adipose tissue after birth. This would result in a selection for cells that have accumulated mitochondrial mutations and explain the higher concentration of mutations in the above-mentioned MSL patient [12]. The well-known association of alcoholism with MSL [2] may augment the growth advantage by influencing the surface receptors of cells thus releasing the adipose tissue from hormonal regulation [16]. Further studies should identify mtDNA mutations in the individual and compare their frequency in normal and affected tissue.

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