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Bioenergetic markers in skin fibroblasts of sporadic ALS and PLS patients

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Abstract

Energy metabolism could influence ALS and PLS pathogenesis and the response to therapy. We developed a novel assay to simultaneously assess mitochondrial content and membrane potential in patients' skin fibroblasts. In ALS and PLS fibroblasts, membrane potential was increased and mitochondrial content decreased, relative to healthy controls. In ALS, higher mitochondrial membrane potential correlated with age at diagnosis and in PLS it correlated with disease severity. These unprecedented findings in ALS and PLS fibroblasts could shed new light onto disease pathogenesis and help developing biomarkers to predict disease evolution and the individual response to therapy in motor neuron diseases.

Keywords

ALS; PLS; Mitochondria; membrane potential; fibroblasts; bioenergetics

Introduction

There is broad consensus in the amyotrophic lateral sclerosis (ALS) field that appropriate biomarkers for sporadic ALS are lacking¹, which is a likely contributor, not only to diagnostic delay, but also to the inability to stratify a heterogeneous population of patients. This deficiency could have played a role in the failure of many therapeutic trials. The availability of useful biomarkers would help with patient stratification and to assign them to appropriate clinical trials; it may also decrease the need for clinical measures as trial endpoints and reduce study length.

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Contributions: Kathryne Kirk, Marilena D'Aurelio, Hibiki Kawamata, Federica Valsecchi: setting up experimental procedures, manuscript and figures preparation; Chris Gennings: statistical analyses; Saba Tadesse: Fibroblast expansion and maintenance; Hiroshi Mitsumoto and Jonathan Hupf: fibroblast randomization and clinical registry; Hiroshi Mitsumoto and Giovanni Manfredi: study design, experimental analyses and manuscript preparation.

A recent study has identified altered plasma metabolites indicative of disrupted mitochondrial function and increased carbohydrate and lipid metabolism in ALS patients². This is consistent with evidence of mitochondrial dysfunction in ALS (reviewed in^{3–5}). However, while mitochondrial function has been investigated in familial ALS with SOD1 mutations, energy metabolism in sporadic ALS (sALS) is largely unexplored.

Living cells are necessary to investigate energy metabolism. Skin fibroblasts are primary cells, which can be used to study mitochondrial energy metabolism. Furthermore, since fibroblasts carry the same genetic composition of neuronal cells, correlations between fibroblast metabolism and disease may suggest a systemic involvement of energy metabolism, involving motor neurons and other cell types that are primary disease targets.

The first goal of this study was to search for bioenergetic parameters that correlate with disease in fibroblasts from sALS and progressive lateral sclerosis (PLS) patients. Second, we wanted to start defining bioenergetic properties that could in the future help stratifying patients, by correlating energy metabolism with disease characteristics, such as age of onset and rate of progression.

Subjects and Methods

Skin biopsies

After informed consent, a punch skin biopsy was obtained from the volar part of the forearm, and skin fibroblasts were cultured as described previously⁶ in DMEM supplemented with 25 mM glucose, 4 mM glutamine, 1 mM pyruvate and 10% FBS. Skin biopsies were coded to protect patients' identity. All cultured fibroblast lines were studied at passages 2–3.

Fifty skin biopsies were from healthy controls (25 males and 25 females); fifty were from patients with definite, probable or possible sALS, randomly selected from 173, and matched for age and gender with the controls; Thirty-five were from clinically “definite” PLS patients (pure upper motor neuron disease, more than 5 years after symptom onset, normal EMG, and no definable causes). All skin samples had been collected by the COSMOS ALS/PLS study group. Table 1 summarizes the characteristics of the subjects whose fibroblasts were utilized for this study.

Bioenergetic measurements

Skin fibroblasts were seeded at the density of 20,000 cell/well in quadruplicate in 48-well tissue culture plates. The next day, cells were loaded with 50 nM tetramethylrhodamine methyl ester (TMRM, 544ex; 590em, Life Technologies) and 25 nM MitoTrackerGreen (MTG, 490ex; 516em, Life Technologies) for 30 minutes at 37°C in phenol-free DMEM containing 5 mM glucose, 4 mM glutamine and 1 mM pyruvate. After washing twice with DMEM, MTG and TMRM fluorescence were simultaneously recorded in a plate reader equipped with a polychromator (Spectramax 5, Hitachi). The protonophor cyanide p-trifluoromethoxyphenylhydrazine (FCCP, 2 µM, Sigma) was then added for 30 min to completely depolarize mitochondria and obtain background TMRM fluorescence, which was subtracted from total fluorescence. MTG and TMRM fluorescence values were

expressed as RFU/mg total cellular proteins (DC Protein Assay, BioRad). All analyses were conducted by operators blind to group identity.

Statistical analyses

Analyses of differences of mitochondrial membrane potential (MMP), mitochondrial mass (MM), and MMP:MM ratio among the three groups (sALS, sPLS, and controls) were by one-way ANOVA with Bonferroni correction for multiple testing. Analyses of covariance tested for differences among the groups in the association between the log of the MMP:MM ratio and various independent clinical parameters.

Results

Bioenergetic markers in sALS and sPLS fibroblasts

Transmembrane potential (MMP) is the energy used by the mitochondria to phosphorylate ATP. TMRM was used to estimate MMP. MTG was used to assess the mitochondrial content or mitochondrial mass (MM), because it accumulates in mitochondria independently of MMP⁷. The co-labeling with the two dyes in control fibroblast mitochondria was demonstrated by confocal microscopy (Fig. 1A). Using the coupled fluorimetric plate-reader assay in control fibroblasts, TMRM fluorescence decreased as expected after membrane uncoupling with FCCP (Fig. 1B, top panel) while MTG fluorescence was insensitive to FCCP (Fig. 1B, bottom panel).

ALS and PLS fibroblasts had significantly higher MMP (Fig. 2A) and lower MM (Fig. 2B) than controls. PLS fibroblasts had higher MMP than ALS fibroblasts. Total TMRM fluorescence depends on MMP but also on MM. Thus, the ratio between TMRM and MTG fluorescence was used to assess MMP relative to MM (MMP:MM ratio). MMP:MM was significantly higher in both ALS and PLS fibroblasts compared to controls (Fig. 2C), and PLS fibroblasts had higher MMP:MM than ALS fibroblasts. Therefore, increased fibroblast MMP, decreased MM, and increased MMP:MM ratio correlated with ALS and PLS disease status.

Correlations between clinical characteristics and bioenergetic markers

As a first step towards testing if patients could be stratified based on fibroblasts bioenergetics we looked at correlations between MMP:MM and patients' characteristics, including gender, age, presentation (bulbar or limb), age of onset, and other characteristics at time of skin biopsy, such as body mass index, ALSFRS-R⁸ score, FVC%, Riluzole treatment. We found a significant interaction between age at time of skin biopsy and MMP:MM in ALS (Fig. 2D), but not in PLS or controls. Since ALS skin biopsies were taken within one year of diagnosis, the finding suggests that higher MMP:MM corresponded to later disease onset. It also indicates that the correlation in the ALS group was not due to normal aging, since no correlation was found in controls. In PLS, but not in ALS, there was an inverse correlation of MMP:MM with ALSFRS-R score (Fig. 2E), indicating that in PLS increased MMP:MM corresponds to more severe disease.

Discussion

Fibroblasts can be obtained from skin biopsies with minimally invasive procedures⁶, which can be repeated during the course of the disease. Interestingly, ALS patients have reduced cutaneous elasticity (leathery skin)⁹. Furthermore, histological studies of skin from sALS patients showed alterations in proteins associated with ALS, such as TDP-43¹⁰, FUS¹¹, and VCP¹², and neuroinflammation, such as TNF- α ¹³ and IL-6¹⁴. In addition, in ALS skin there was elevation of matrix metallo-proteases 2 and 9¹⁵, with the latter playing a role in selective motor neuron degeneration¹⁶. These findings support the use of fibroblasts for derivation of motor neurons and glial cells¹⁷. To this end, astrocytes differentiated from neural progenitor cells derived from ALS skin fibroblasts were toxic for motor neurons¹⁸. This body of evidence provides a rationale for utilizing fibroblasts to investigate bioenergetic biomarkers of sALS.

We have performed a study of unprecedented size on mitochondrial bioenergetics in sPLS and sALS fibroblasts, using a simple and reproducible high throughput method. The bioenergetic changes in ALS and PLS fibroblasts and the correlations with disease characteristics evidenced similarities and differences between these two motor neuron diseases, which have different neuronal involvement and clinical outcomes. MMP was higher in PLS than ALS; an age correlation was observed only in ALS, while the correlation with disease severity only in PLS. One caveat is that the diagnosis of PLS is largely based on clinical exclusion criteria. Clinically diagnosed PLS occasionally prove to be ALS at autopsy, and autopsies are extremely rare. We defined PLS clinically to the best of our capabilities, but we cannot completely exclude the possibility that some PLS cases were mild ALS.

The mechanisms underlying the bioenergetic changes in sALS and sPLS fibroblasts and their involvement in disease pathogenesis remain to be clarified. Our hypothesis is that ALS and PLS fibroblast mitochondria are less energy efficient than controls, leading to increased reliance on glycolysis for ATP production. This hypothesis is consistent with findings in I113T mutant SOD1 fibroblasts¹⁹. It could be speculated that sALS patients with higher MMP have later disease onset, because their mitochondria work harder to compensate a metabolic defect. Furthermore, sPLS has a milder disease course and higher MMP. Thus high MMP could be an indicator of metabolic dysfunction, but also a protective factor. Further studies of energy metabolism will contribute to clarify the biochemical mechanisms underlying these changes.

It also remains to be determined if bioenergetic changes occur in fibroblasts harboring mutations in genes associated with familial ALS. Studying differences and similarities among familial and sporadic forms of ALS could help to better understand the common denominators of motor neuron diseases.

Lastly, it will be important to assess if metabolic changes in ALS and PLS fibroblasts reflect systemic changes in cell types that are primarily involved in the disease, such as motor neurons and astrocytes. Clearly, if increased MMP represents an attempt to compensate for inefficient mitochondrial ATP generation, high energy consuming cells, such as motor

neurons, could be exquisitely sensitive. One potential approach to address this problem will be to derive motor neurons from patients' fibroblasts and study their bioenergetics in comparison with that of the parental fibroblasts.

In conclusion, this study has identified changes in the bioenergetic settings in sALS and sPLS. While the patient cohort investigated so far is a first step in the process of stratifying sALS and sPLS patients, based on these intriguing results, it is plausible that extending mitochondrial studies to larger cohorts of patients' fibroblasts will generate new tools for patient stratification and help building useful criteria for patient selection in clinical trials.

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Figure 1.

A. Representative confocal microscopy images of a control skin fibroblast mitochondria co-stained with TMRM and MTG. **B.** Fluorimetric measurement of changes (% of initial value) in MMP and MM signals in response to 2 μM of the uncoupler FCCP for 30 minutes. While 80% of TMRM fluorescence is lost in response of depolarization (top panel), MTG fluorescence is unchanged (bottom panel).

Figure 2.

A. Measurements of mitochondrial membrane potential (MMP) and **B** mitochondrial mass (MM). ALS n=50, PLS n=35, controls (CTL) n=50. In A and B, MMP and MM are expressed as relative fluorescence units (RFU)/mg of cellular protein. **C** Ratio of MMP:MM. Data are average values $\pm$ SEM. Analyses of differences were by one-way-Anova with Bonferroni correction; p values are indicated below each panel; * indicates statistically significant differences vs. CTL. **D.** Interaction between age at time of skin biopsy and log(MMP:MM) in ALS (p=0.014). As age increased for ALS patients, the ratio also increased (positive slope; p=0.024); the mean ratio did not change with age for PLS (p=0.671) and CTL (p=0.930). **E.** Interaction between ALSFRS-R and log(MMP:MM). There was a significant negative interaction for PLS patients (Figure 6; p=0.025), but not for ALS patients (p=0.623).

Table 1

Patients whose skin fibroblasts were studied. BMI, Body Mass Index; FVC%, residual forced vital capacity; ALSFRS-R, ALS functional rating scale; Riluzole, number of patients who were receiving treatment at the time of skin biopsy. For Age of onset and age at biopsy, ALSFRS-R, BMI, and FVC% numbers represent averages $\pm$ standard deviations.

	ALS	PLS	CTL
Number	50	35	50
Sex	25 F, 25 M	16 F, 19 M	25 F, 25 M
Age of Onset	59 $\pm$ 10.23	52 $\pm$ 8.33	N/A
Age at Biopsy	60 $\pm$ 10.14	59 $\pm$ 8.62	60 $\pm$ 8.55
Presentation	16 Bulbar, 34 Limb	9 Bulbar, 25 Limb	N/A
ALSFRS-R	33 $\pm$ 8.92	33 $\pm$ 5.91	N/A
BMI	26 $\pm$ 5.7	27 $\pm$ 4.1	N/A
FVC %	75 $\pm$ 27.6	89 $\pm$ 21.7	N/A
Riluzole	27	1	0