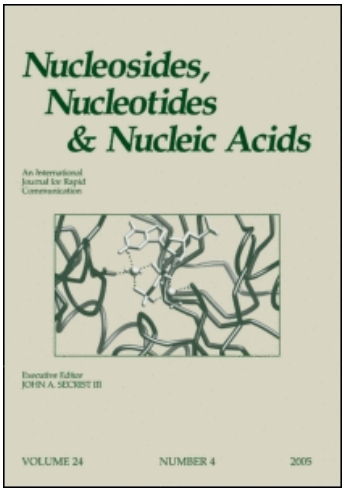

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Mitochondrial Function Dependent Proliferation Assay for the Diagnosis of Mitochondrial Disorders in Human Fibroblasts

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ABSTRACT

A media has been developed which enables the assessment of mitochondrial function in fibroblasts by measuring proliferation as an end point.

Key Words: Fibroblast proliferation assay; Mitochondrial disorders; Inosine as pentose source.

INTRODUCTION

Mitochondrial disorders are difficult to diagnose due to confounding factors including maternal transmission, heteroplasmy, differential tissue severity and the combined contribution of nuclear and mitochondrial gene products to organelle function. The analysis of disorders of oxidative phosphorylation has relied upon complex methodologies for measurement of respiratory chain function by polarography

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or biochemical assay[1,2] and increasingly specific DNA based diagnostic tests. The choice of patient cell or tissue sample varies from lymphocytes and fibroblasts to muscle or organ biopsy with further distinction regarding the use of permeablized cells or mitochondrial preparations from fresh or frozen tissue. The method presented here using cultured fibroblasts provides a comparatively simple assay for the global assessment of mitochondrial integrity and function.

MATERIALS AND METHODS

Fibroblasts are established from biopsy in minimum essential Eagle's media (Gibco) supplemented with 2 mM glutamine, 1 mM pyruvate, 0.2 mM uridine and 10% fetal calf serum. Uridine has previously been indicated to preserve respiratory chain deficiencies in cultured fibroblasts.[3] For assay of mitochondrial function, established fibroblasts are seeded at equivalent density into a mitochondrial function sparing media and a mitochondrial function requiring media. The mitochondrial function sparing media is the same as that in which the cells were established minus uridine. The mitochondrial requiring media is the same as the sparing media minus glucose and supplemented with 0.5 mM inosine. Cells are photographed at 24 hour intervals with an inverted Zeiss microscope equipped with a Cannon D60 digital camera.

RESULTS AND DISCUSSION

Glutamine is known to be a major and adequate energy source for human cells in culture.[4–6] In the absence of glucose and presence of glutamine there remains a pentose requirement which can be met by inosine.[5,6] For avian reticulocytes only inosine plus glutamine sustained ATP in the absence of glucose, whereas uridine, glutamate or pyruvate were ineffective.[7] Although the ribose moiety of inosine may be converted to glycolytic intermediates, it is also clear that nucleotide synthesis is supported via the ribose moiety as a precursor for PP-ribose-P.[5–8]

Our initial objective was to develop a media which sustained proliferation for normal fibroblasts when dependent upon oxidative phosphorylation for their energy requirements. The metabolic requirements for cells cultured in the absence of glucose and the presence of glutamine as an oxidative energy source were shown to be met by inosine, presumably as a pentose phosphate source. In the absence of glucose and the presence of glutamine, inosine enables cell proliferation whereas neither hypoxanthine nor uridine supported fibroblast proliferation under the conditions used (Fig. 1). These findings indicate that the phosphorolysis of inosine to hypoxanthine and ribose-1-phosphate by purine nucleoside phosphorylase is required for provision of the pentose-phosphate as opposed to the purine base. The inability of uridine to support growth indicates that insufficient pentose-phosphate sparing is attained via conversion of uridine to UMP or by cleavage to ribose-phosphate. In contrast, for mouse Ehrlich ascites tumour cells, uridine was able to support proliferation in the absence of glucose by one of these pathways.[9] Fibroblasts cultured in the absence of glucose and the presence of inosine were able to proliferate when provided with glutamine as an oxidative energy source, but not with either glutamate or asparagine (Fig. 2).

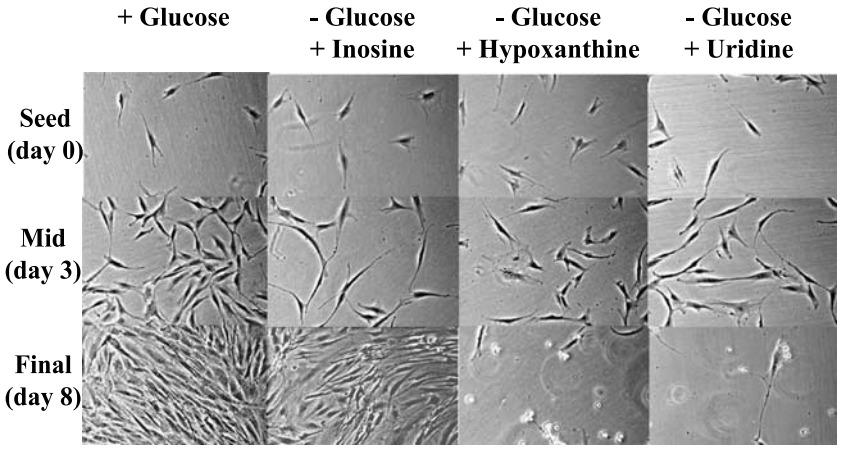

Figure 1. Demonstration of the inosine requirement for fibroblasts cultured in the absence of glucose and the presence of glutamine. Cells were cultured with glutamine, 2 mM, and as indicated in the presence or absence of: glucose, 5.5 mM; inosine, 0.5 mM; hypoxanthine, 0.1 mM; uridine, 0.2 mM.

Having determined conditions for which fibroblasts with normal mitochondrial function are able to proliferate while dependent upon oxidative phosphorylation, these conditions were applied to a series of fibroblast lines with known mitochondrial disorders. Fibroblasts were subcultured at equal density on Day 0 in both mitochondrial sparing and mitochondrial function requiring media and photographed daily until the sparing culture had reached confluence. The results for four such lines show that cells

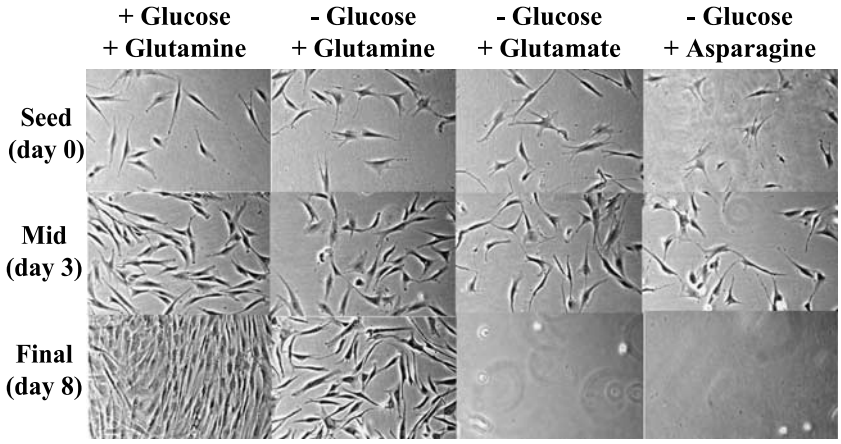

Figure 2. Demonstration of glutamine requirement as an oxidative energy source for fibroblasts cultured in the absence of glucose and presence of inosine. Cells were cultured with 0.5 mM inosine and as indicated in the presence or absence of glucose, 5.5 mM; glutamine, 2 mM; glutamate, 2 mM; asparagine, 2 mM.

proliferated to confluency in the mitochondrial function sparing media, but in each case there was essentially a complete attrition of cells in the mitochondrial function requiring media for a Leigh disorder/Surf+, complex I deficiency, NARP T8993G mutation, and cytochrome C oxidase deficiency (Fig. 3).

We also examined a fibroblast line characterized as having a partial deficiency of complex IV by biochemical assay and an apparent complete deficiency by polarographic measurement. In the mitochondrial function requiring media, this cell line showed significant growth retardation and loss of cells by day 3, but a substantial outgrowth on day 6 (Fig. 4). Together these findings suggest the presence of a heterogeneous cell population having a subset which retained the capacity to proliferate under the conditions requiring oxidative metabolism.

Although no single tissue provides a definitive assessment of mitochondrial function, the method described here may provide an initial screening tool for mitochondrial disorders which is readily accomplished by comparative growth under mitochondrial sparing and mitochondrial function demanding conditions. The mitochondrial function dependent proliferation assay has been incorporated into our

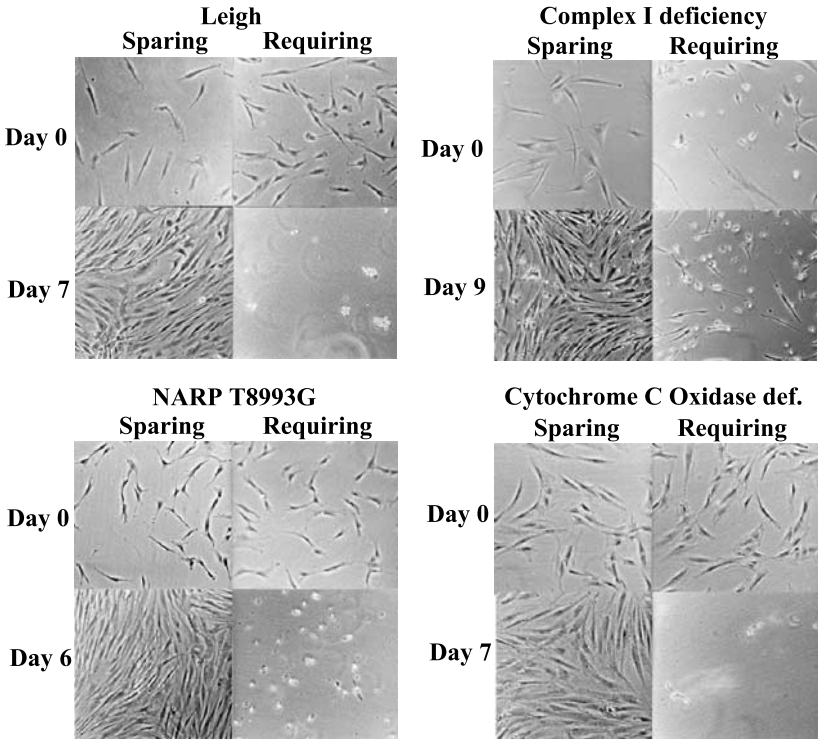

Figure 3. Application of the mitochondrial dependent proliferation assay to fibroblasts having mitochondrial mutations. Cells were seeded at equal densities in mitochondrial function sparing and requiring media as described in Methods and allowed to proliferate to confluency for the sparing conditions. Fibroblast lines are as identified with each set.

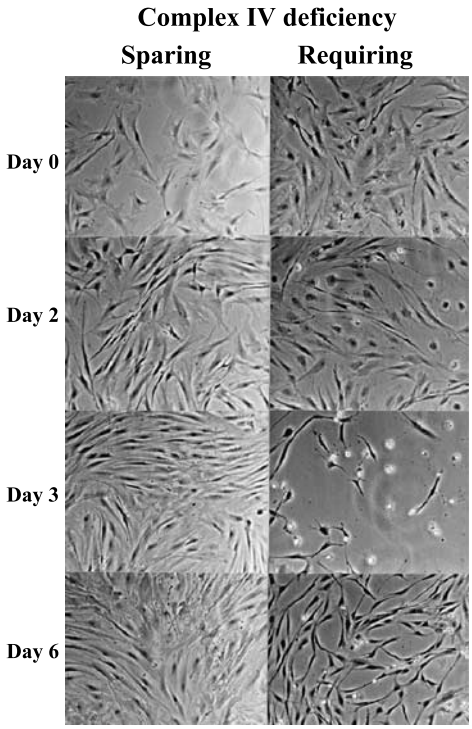

Figure 4. Application of the mitochondrial dependent proliferation assay to a partially deficient complex IV fibroblast line. Cells were seeded at equal densities in mitochondrial function sparing and requiring media as described in Methods.

protocol for the establishment and testing of fibroblasts submitted for assessment of mitochondrial disorders.

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