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Effects of alginate encapsulation on mitochondrial activity

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The long-term objective of our research is to study the biochemical consequences of primary genetic defects of the Pyruvate Dehydrogenase Complex, a key mitochondrial enzyme complex, by NMR spectroscopy. An established method to obtain energetic and metabolic information from intact cells involves the use of ^{31}P and ^{13}C NMR spectroscopic techniques. NMR spectra from live and fully functional cells can be obtained from cells encapsulated within alginate beads and maintained in a perfusion bioreactor throughout the NMR experiment. However, before spectroscopic studies can commence, the effects of alginate encapsulation on the general metabolism and mitochondrial activity of fibroblasts need to be determined. In this study we report glucose consumption and flow cytometry measurements (with the fluorescent markers MitoTracker GreenFM™ and Nonyl-acridine Orange™ to determine the mitochondrial status and mass) of healthy human fibroblasts encapsulated in a mannuronic acid-rich alginate matrix. The results show that alginate encapsulation of fibroblasts does not affect the glucose consumption, the mitochondrial integrity, or the mitochondrial mass during 21 days of *in vitro* culture.

1. Introduction

Alginate is a family of unbranched polymers composed of 1,4-linked β-D-mannuronic and α-L-guluronic acid residues in varying proportions, sequence and molecular weight. The gelation of alginate occurs when multivalent cations (usually Ca^{2+}) interact with blocks of guluronic residues between different chains of alginate, creating a three-dimensional network [1]. The strength of this network depends on the overall fraction of guluronic acid residues, the molecular weight of the polymer, and the Ca^{2+} ion concentration at the time of gelation [2]. The physical properties of alginate gels vary widely depending on their chemical composition. Alginates possessing a high guluronic acid content develop stiffer, more porous gels which maintain their integrity for longer periods of time, whereas alginates rich in mannuronic acid have reciprocal properties.

Given the variety of physical properties associated with alginate composition, it is reasonable to hypothesize that encapsulated cells might be affected by changes in the composition of alginate. In our laboratory, we have explored these effects by entrapping βTC3 murine insulinoma cells within alginates of varying molecular weight, structure, and concentration [3–6]. Through these studies, we demonstrated that alginates rich in guluronic acid hinder cell growth and overall metabolic activity of the entrapped cells. Conversely, alginates rich in mannuronic acid allowed entrapped cells to grow at an exponential rate, until the microbeads broke due to the high cell density. Furthermore, the growth patterns of the βTC3 cells within the mannuronic rich alginates and the guluronic rich alginates differ significantly: from an o-ring pattern to a clustering pattern, respectively. Although these studies described the effects of alginate encapsulation on the growth and gross metabolic activity of the cells, they did not detail the effect of encapsulation on specific organelles critical to the survival and function of the cells.

The mitochondrion is an organelle found in the cytoplasm of eukaryotic cells. Most mammalian cells contain hundreds of mitochondria which are responsible for key cellular processes such as oxidative metabolism, energy production, protection from reactive oxygen species as well as protection from necrosis and apoptosis. The mitochondrion is also the organelle involved in an array of rare disorders that relate to specific defects in metabolism. Genetic mutations responsible for these defects can arise in the nuclear or mitochondrial DNA (mtDNA). Mutations of the mtDNA were first associated with disease in 1988 by Wallace *et al.* [7] and Holt *et al.* [8]. Since then, genetic mutations resulting

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in defective mitochondrial proteins have been associated with a variety of diseases [9] ranging from aging to diabetes to cancer.

The traditional approach to diagnose inborn errors of metabolism has relied on: (1) clinical suspicion of a disease phenotype; (2) quantification of readily measurable, but relatively nonspecific, surrogate disease markers such as the blood lactate concentration; and (3) enzymological and/or molecular genetic techniques that try to identify the primary defect. In the context of mitochondrial disorders, and despite current state-of-the-art methods, the etiology of disease remains unresolved in a substantial number of patients with unequivocal clinical evidence of a congenital disorder possibly involving mitochondrial energetics. Moreover, probative investigation of the etiopathology of the disease typically begins and ends with the discovery and characterization of the defective gene or protein without determining the functional biochemical consequences upstream or downstream of the defect. For example, little is known of the quantitative or qualitative consequences of pyruvate dehydrogenase complex (PDC) deficiency on vital processes of bioenergetics, such as the flux through the citric acid cycle, fatty acid synthesis, or the overall intracellular energy charge.

The long-term objective of our research is to study the biochemical consequences of PDC defects through NMR spectroscopic techniques on living cells. Two important advantages of using NMR on living systems are that it is non-invasive and that longitudinal studies on the same sample can be obtained. ^{31}P NMR spectroscopy can yield information on energy stores in the cell (e.g., ATP, phosphocreatine) as well as inorganic phosphate and phosphomono- and phosphordiester levels within the cells. ^{13}C spectroscopy, in conjunction with feeding cells a ^{13}C-labeled fuel source (e.g. glucose) can yield important metabolic information such as integration of the label into key metabolites (e.g., amino acids such as alanine, glutamate and aspartate), and activity of enzyme systems feeding the citric acid cycle.

An established method to obtain NMR spectra from intact cells is to encapsulate the cells within alginate beads, place the beads in a perfusion bioreactor that is subsequently positioned within the magnet's isocenter, and perfuse them [10–14], thereby maintaining the cells alive and functional for the duration of the experiment. Before these studies should commence, the effects of alginate encapsulation on the general metabolism and mitochondrial activity of fibroblasts need to be determined. In the present study we report flow cytometry measurements on the mitochondrial status and mass in healthy human fibroblasts encapsulated in mannuronic acid-rich alginate beads. These measurements were performed 24 h post-encapsulation and after 21 days of *in vitro* culture.

2. Methods

2.1. Fibroblast cultures

Human skin fibroblasts were obtained with institutional review board approval from healthy controls. The cells were cultured as monolayers in T-flasks in completed Dulbecco's Modified Eagle's Medium (DMEM). The medium contains 25 mM glucose and was supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin solution (10,000 U of penicillin and 10 mg/ml of streptomycin), 1% insulin-transferrin solution (0.5 mg/ml), 0.2% fibroblast growth factor solution (1 μg/ml), and 1% L-glutamine solution (0.1 M). Cultures were maintained at 37 °C in a humidified 5% CO_2 /95% air atmosphere. Fibroblasts were confluent every 10–14 days at which point they were propagated with a 1:2 split. Once sufficient flasks had reached confluence the cells were trypsinized, counted and dispersed in a 2% alginate solution for encapsulation.

2.2. Alginate encapsulation

Freshly trypsinized healthy human fibroblasts were suspended in a 2% sodium alginate solution at a density of 1.63×10^6 cells/ml alginate. The alginate used in this study had a high mannuronic acid content (LVM type alginate from NovaMatrix, Oslo, Norway). Specifically, the guluronic to mannuronic ratio for the alginate used in these experiments was 38%/62% guluronic/mannuronic content with 18% of GG blocks. Spherical alginate beads (0.7 mm in diameter) were generated by dropping alginate into 100 mM $CaCl_2$ using an electrostatic bead generator (NISCO, Zurich, Switzerland). Approximately 3 ml of these cell-containing beads were placed in T-75 flasks, fed with 20 ml of fully supplemented DMEM and maintained on a rocker within a temperature-regulated humidified incubator.

2.3. Analytical techniques

The metabolic state of the encapsulated cells was assessed by monitoring their glucose consumption rate through the lifetime of the culture. Media samples were drawn from the flask both immediately after feeding the cells and 24 h later. Glucose concentrations of the media samples were measured using a VITROS DT60 II Bioanalyzer (Ortho-Clinical Diagnostics, Rochester, NY). The rate of glucose consumption (GCR) was calculated from the change in the amount of glucose (amount = volume $\times$ concentration) during the incubation period (20–24 h). This rate was normalized to a unit of 10^5 cells.

2.4. Mitochondrial assays

The general status of mitochondria was assessed with the aid of flow cytometry techniques involving the use of the fluorescent dyes MitoTracker Green FM™ (Molecular Probes, Eugene, OR), and Nonyl-acridine Orange™ (Molecular Probes, Eugene, OR). Mitotracker GreenFM™ introduced in nanomolar concentration, stains the mitochondria and exhibits a bright fluorescein-like fluorescence. It accumulates in the lipid environment of the mitochondria regardless of mitochondrial membrane potential and has been used as a tool to determine the abundance and mass of mitochondria within cells. Alternatively, Nonyl-acridine Orange™ binds to cardiolipin in all mitochondria regardless of their energetic state (membrane potential

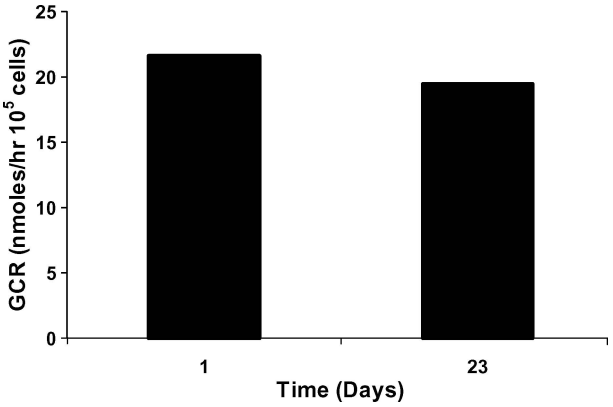

Figure 1 Glucose consumption rate of an alginate-encapsulated healthy human fibroblast culture at days 1 and 23 after encapsulation. These rates represent the average consumption of glucose over a 24 h period referenced to the initial number of cells present at the time of entrapment. The data do not indicate any gross apparent differences in glucose consumption with prolonged culture.

and proton gradient) and has been used to determine mitochondrial differences between apoptotic and non-apoptotic cells. To perform flow cytometric measurements, fibroblasts encapsulated in alginate beads were released from the alginate matrix by treatment for 10 min in a solution of isotonic 50 mM sodium citrate. Similarly, trypsinized cells from monolayer cultures were also treated for 10 min with the sodium citrate solution and used as controls. Approximately 10^5 fibroblasts were incubated for 30 min at 37 °C in completed DMEM containing either 400 nM MitoTrackerGreenFM™ or 1.5 mM of Nonyl-acridine Orange™ . Following the incubation, the cells were spun down, washed twice with PBS and resuspended in 1 ml of PBS. Aliquots of 20,000 cells were analyzed using a FacScan (BD biosciences, Lincoln Park, NJ) equipped with a single 488 nm argon laser. The wavelengths of absorption and emission are as follows. Mitotracker GreenFM™ ($l_{Ab} = 490$ nm, $l_{Em} = 516$ nm) and Nonyl-acridine Orange™ ($l_{Ab} = 495$ nm, $l_{Em} = 519$ nm). The data obtained were analyzed with WinMDI version 2.5.

3. Results and discussion

Healthy human fibroblasts encapsulated in alginate beads were maintained *in vitro* for 23 days. During this period the metabolic activity of the cells was assessed periodically by measuring the rate of glucose consumption. Fig. 1 is a bar graph showing no statistical significant difference between the rate of glucose consumption measured on Day 1 and that measured on Day 23. These results indicate that the cells were metabolically active and their values maintained constant throughout the 23 days of culture. Although this observation is in stark contrast with the continuous increase in glucose consumption reported with the βTC3 murine insulinoma cells encapsulated in the same alginate matrix [3, 4, 6], it is not surprising given the inherently slower doubling times of fibroblast cells, and suggests that the entrapped cell population is stable.

The status of the mitochondria with the encapsulated cells was assessed three times during this study: once at the beginning of the experiment before the cells were encapsulated, once more within 24 h of encapsulation, and once at the conclusion of the experiment after 21 days of culture. Fig. 2 shows representative flow cytometric measurements for MitoTracker GreenFM™ and Nonyl-acridine Orange™ of freshly trypsinized fibroblasts and alginate entrapped fibroblasts cultured for 21 days. The data show no statistical differences between the status and mass of mitochondria between freshly trypsinized and encapsulated fibroblasts regardless of the fluorescent dye employed. The same pattern

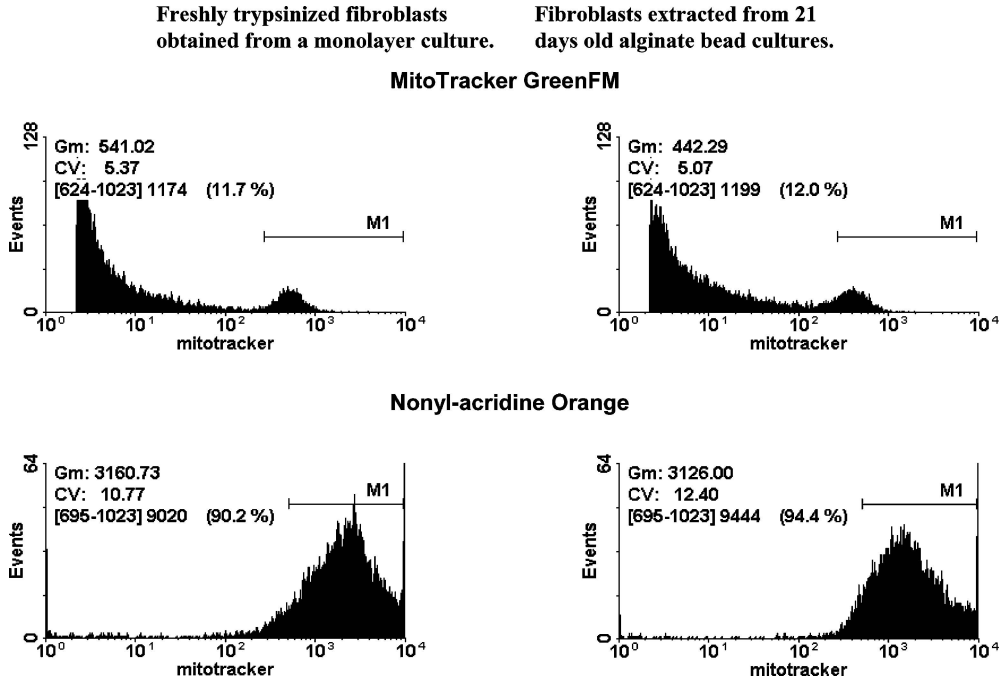

Figure 2 Flow cytometry data from freshly trypsinized fibroblasts and fibroblasts extracted from 21 days old alginate bead cultures. Note that there are no statistical differences between the distributions of the two cell populations regardless of the fluorescent stain used.

was observed from fibroblasts encapsulated for only 24 h (data not shown). This observation is significant because it clearly demonstrates that alginate encapsulation does not have a detrimental effect on either mitochondrial status or mass. It also demonstrates that through alginate encapsulation, one can maintain human fibroblast cultures unaffected for at least a period of 21 days, opening up the potential for longitudinal studies.

4. Conclusions

Our data show that alginate encapsulation of healthy human fibroblasts does not have a detrimental effect on the metabolic activity of the cells or on their mitochondrial content and mass. Specifically, alginate encapsulation allows fibroblasts to be maintained fully functional in a 3-dimensional matrix for a period of at least 23 days, permitting the use of NMR spectroscopic studies to observe longitudinal changes in bioenergetics and metabolism in response to potential modifiers of metabolism and different physiological conditions.

Acknowledgments

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References

1. G. T. GRANT, E. R. MORRIS, D. A. REES, P. J. C. SMITH and D. THOM, *FEBS Lett.* **32** (1973) 195.
2. A. MARTINSEN, G. SKJAK-BRAEK and O. SMIDSROD, *Biotechn. Bioengng.* **33** (1989) 79.
3. C. K. STABLER, C. WILKS, A. SAMBANIS and I. CONSTANTINIDIS, *Biomaterials* **22** (2001) 1301.
4. I. CONSTANTINIDIS, I. RASK, R. C. LONG JR. and A. SAMBANIS, *ibid.* **20** (1999) 2019.
5. C. L. STABLER, A. SAMBANIS and I. CONSTANTINIDIS, *Annals New York Acad. Sci.* **961** (2002) 130.
6. N. E. SIMPSON, C. L. STABLER, A. SAMBANIS and I. CONSTANTINIDIS, *Biomaterials.* **25** (2004) 2603.
7. D. C. WALLACE, G. SINGH, M. T. LOTT, J. A. HODGE, T. G. SCHURR, A. M. LEZZA, L. J. ELSAS 2nd and E. K. NIKOSKELAINEN, *Science* **242** (1988) 1427.
8. I. HOLT, A. E. HARDING and J. A. MORGAN-HUGHS, *Nature* **331** (1988) 717.
9. D. JOHNS, *New England J. Med.* **333** (1995) 638.
10. I. CONSTANTINIDIS and A. SAMBANIS, *Biotechn. Bioengng.* **47** (1995) 431.
11. K. K. PAPAS, R. C. LONG JR., I. CONSTANTINIDIS and A. SAMBANIS, *Biocheml. J* **326** (1997) 807.
12. *Idem.*, *Biotech. Bioengng.* **66** (1999) 219.
13. K. K. PAPAS, R. C. LONG JR., A. SAMBANIS and I. CONSTANTINIDIS, *Cell Transplant.* **9** (2000) 415.
14. R. C. LONG JR, K. K. PAPAS, A. SAMBANIS and I. CONSTANTINIDIS, *J. Magn. Reson.* **146** (2000) 49.

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