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Monolayer expansion induces an oxidative metabolism and ROS in chondrocytes

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ABSTRACT

This study tests the hypothesis that articular chondrocytes shift from a characteristically glycolytic to an oxidative energy metabolism during population expansion in monolayer. Bovine articular chondrocytes were cultured in monolayer under standard incubator conditions for up to 14 days. Cellular proliferation, oxygen consumption, lactate production, protein content, ROS generation and mitochondrial morphology were examined. Lactate release increased ~5-fold within 1 week, but this was limited to ~2-fold increase when normalized to cellular protein content. By contrast, per cell oxidative phosphorylation increased 98-fold in 1 week. The increase in oxidative phosphorylation was evident within 24 h, preceding cell proliferation and was associated with augmented reactive oxygen species generation. The autologous chondrocyte implantation procedure requires 14–21 days for population expansion. The alterations in metabolic phenotype we report within 7 days *in vitro* are thus pertinent to autologous chondrocyte implantation with significant implications for the chondrocyte functionality.

Articular cartilage is a tissue with limited intrinsic repair capacity and autologous chondrocyte implantation techniques have been developed for the treatment of cartilage defects [1,2]. The cells are derived from a biopsy of cartilage removed from low-load bearing regions of the joint [1,3,4]. Typically, a 10- to 20-fold expansion is required to obtain sufficient cell numbers from the cartilage biopsy for therapeutic use, which is achieved over a 2–3 week period by monolayer culture under atmospheric oxygen tension [1,2,4]. *In vitro* culture is also used for research purposes, to expand cell populations from rare human material.

Articular chondrocytes are well adapted to the low oxygen environment which they inhabit *in vivo* and derive most energy from substrate level phosphorylation during glycolysis. This adaptation is not mediated via a positive Pasteur effect, as occurs during exposure of aerobic cell types to hypoxia [5]. Rather, chondrocytes retain high levels of glycolysis even in an aerobic environment, a phenomenon known as the Warburg effect [6,7]. The retention of a glycolytic metabolism in an aerobic environment is a trait shared by a few other cell types, including tumor cells and embryonic stem cells. Although it has long been established that chondrocytes exhibit low oxygen consumption rates compared to most cell types [6,8], we have identified from the literature a marked discrepancy between oxygen consumption by chondrocytes *in situ* and immediately following isolation when compared to the much higher consumption by chondrocytes which have been cultured in monolayer [9].

We hypothesize that articular chondrocytes undergo a fundamental switch in their energy metabolism following introduction to the monolayer culture environment, from a predominantly glycolytic metabolism to an oxidative one. An oxidative metabolism is associated with increased generation of reactive oxygen species (ROS), with up to 5% of oxygen consumption in the electron transport chain diverted into superoxide production [10]. In turn, exposure to ROS has already been demonstrated to promote the onset of premature proliferative senescence [11–14], with clearly detrimental implications for the long-term expansion potential of the cells. It is also important to understand how the cellular oxygen consumption rates change with time *in vitro*, as this will determine the local oxygen concentration on re-introduction to a 3D environment [15]. Oxygen concentration has been demonstrated to be a key determinant for the expression of ECM molecules, which is vital for the regeneration of cartilage tissue [16,17]. In particular anoxia, though tolerated by chondrocytes, severely inhibits their metabolism [18].

In the present study we test the above hypothesis, monitoring the metabolism of primary chondrocytes for the first 14 days in monolayer following their isolation from the joint. In addition we ascertain the mechanism behind the metabolic shift including the analysis of the cellular oxidative capacity and relative respiratory reserve as well as analysis of ROS generation. The elucidation of these processes is vital for establishing culture protocols to achieve the full expansion potential of autologous cell populations

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and to optimize the longevity and functionality of repair tissue derived from these cells.

Materials and methods

Cell source and preparation. Cartilage from bovine metacarpal-phalangeal joints was incubated at 37 °C for 1 h in Dulbecco's modified Eagle's medium (DME) supplemented with 20% (v/v) foetal calf serum, 2 μM L-glutamine, 5 μg mL^{-1} penicillin, 5 μg mL^{-1} streptomycin, 20 mM HEPES buffer, and 0.85 μM L-ascorbic acid (DME+20% FCS, all Sigma Chemical Co., Poole, UK) + 700 unit mL^{-1} pronase (Sigma, Poole, UK) and for a further 12 h with 100 unit mL^{-1} collagenase type XI (Sigma, Poole, UK). The chondrocytes were passed through a 70 μm pore size cell sieve (Falcon Ltd, Oxford, UK) and washed twice in DME + 20% FCS. For each experiment, the cells from a total of three animals were pooled.

Monolayer culture. About 4×10^6 freshly isolated chondrocytes were introduced into each 175 cm^2 culture flask, providing a final seeding density of 2.3×10^4 cells cm^{-2}. Each flask was supplied with 45 mL DME + 10% FCS, replenished every 3 days. Any cells in the spent media were recovered by centrifugation and reintroduced to the flasks. The cells were passaged after 10 days, using trypsinisation.

Cell proliferation and morphology. The cells were released from the culture flasks by trypsinisation (5 min). The cell yield was recorded, using haemocytometer. An aliquot of cells was incubated with the cytoplasmic stain, 5 μM calcein AM (Invitrogen Molecular Probes, Paisley UK) for 15 min such that the diameter of the cells, which resumed a spherical morphology in suspension, could be measured from epifluorescent microscope images (Nikon TE200, 60× objective). About 1×10^6 cells mL^{-1} were stained with 200 nM mitotracker green (Invitrogen, Molecular Probes, Paisley UK) for 30 min at 37 °C. The cells were incubated on glass overnight prior to collecting images by epifluorescent microscopy (Nikon TE200, 60×). The protein content in 2×10^6 cells was measured using the bicinchoninic acid protein assay (Pierce, Rockford, Ill).

Oxygen consumption measurement. The cells were suspended at 4×10^6 cells mL^{-1} in DME + 10% FCS that had been equilibrated with a standard incubator atmosphere. Aliquots were transferred to the wells of an O_2 biosensor plate (BD Biosciences, Oxford, UK) in triplicate with 200 μM and 0 μM O_2 reference conditions and [O_2] recorded from the sealed plate at 10 min intervals, as described earlier [9]. On days 0 and 7 additional samples were tested as above, but in the presence of either 3 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or 2 μg mL^{-1} oligomycin to determine maximal and non-F_1F_0 mediated oxygen consumption rate, respectively.

Measurement of lactate release. Lactate released by the cell suspension was determined from media samples. βNAD$^+$ was reconstituted with 0.2 mL glycine buffer, 0.4 mL water and 0.01 mL LDH suspension per mg βNAD (Sigma, Poole, UK). 280 μL of the reconstituted βNAD$^+$ reagent were added to 20 μL of each sample and standards in duplicate and incubated at 37 °C for 30 min. The concentration of lactate in the samples was calculated by comparison of the absorbance at 340 nm to lactate standards ranging in concentration from 0 to 5 mM.

Measurement of reactive oxygen species (ROS) using H_2-DCF-DA. On diffusion into the cell, H_2-DCF-DA is a substrate for intracellular esterases that hydrolyze the molecule to form the anionic, membrane impermeable, non-fluorescent molecule, H_2-DCF. On oxidation by intracellular ROS, H_2-DCF forms the fluorescent DCF molecule. Cell suspensions at 1×10^6 cells mL^{-1} were loaded with 20 μM H_2-DCF-DA in PBS + 1 g L^{-1} glucose (Invitrogen Molecular Probes, Paisley UK) by incubation for 40 min at 37 °C. The cells were washed twice and the rate of fluorescent DCF generation in 300 μL aliquots recorded using a fluorimeter (ex 485 nm, em 520 nm). The experiment was repeated with the control Carboxy-2′,7′-dichlorofluorescein diacetate (C369, Invitrigen Molecular Probes, Paisley UK). C369 is also a substrate for intracellular esterases, but its fluorescence is not dependent on oxidation by ROS.

Results

Cell proliferation and morphology

After a lag time of approximately 3 days the cells adopted a fibroblastic morphology and began to proliferate, with 6.4×10^6 cells $flask^{-1}$ at day 7 and 25.6×10^6 cells $flask^{-1}$ at day 14. Both the mean and variability of the cell diameters increased with culture. Freshly isolated cells and those recovered to suspension following 7 days in monolayer culture had mean cell diameters of 10.2 and 15.1 μm, respectively, equating to cell volumes of 552 and 1789 μm^3. Mitotracker green was used as a membrane-potential independent probe for mitochondria. Fig. 1 shows that the threadlike mitochondria appear to be evenly distributed throughout the cytoplasm, without preferential location in the perinuclear region. Cells that had been cultured in monolayer for 7 or 14 days were significantly larger with markedly increased mitochondrial content when compared to freshly isolated cells. The total protein content of the cells increased throughout culture, rising from an initial value of 59 to 157 pg $cell^{-1}$ after 7 days, reflecting the ~3-fold increase in cell volume.

Lactate release

The release of lactate into the culture medium over a 30 min time interval was used as a measure of glycolytic rate as this cell type as no demonstrable lactate utilization [19]. Fig. 2A indicates that the lactate release increased almost 5-fold over the first 7 days in monolayer culture, from 130 ± 28 to 600 ± 131 f mol $cell^{-1}$ h^{-1} (t-test $p < 0.01$). Fig. 2A indicates that this effect was initiated as early as the first day *ex-vivo*, with a 2-fold increase in lactate production within the first day in monolayer. However, when normalized to the protein content of the cells, lactate release doubled from 2.19 to 3.92 pmol lactate ng $protein^{-1}$ h^{-1} within the first day, but plateau thereafter (Fig. 2C).

Oxygen consumption rate

Bovine articular chondrocytes exhibited an oxygen consumption rate of 1.34 ± 0.10 f mol $cell^{-1}$ h^{-1} immediately after isolation. After 1 day oxygen consumption was 2.38 ± 0.40 f mol $cell^{-1}$ h^{-1}, significantly (t-test, $p<0.01$), greater than for freshly isolated chondrocytes (Fig. 2B). By day 7 values had attained 83.15 ± 8.38 f mol $cell^{-1}$ h^{-1}. By contrast to the steady values for lactate release, cell-protein normalized oxygen consumption rates increased throughout culture (Fig. 2C), rising 31.4-fold from initial values of 23 ± 1.6 to 712 ± 163 f mol oxygen ng $protein^{-1}$ h^{-1} at day 14.

Mitochondrial oxidative capacity

The oxygen consumption of chondrocytes was further characterized using the pharmacological agents CCCP and Oligomycin (Fig. 3). Oligomycin inhibits the F_1F_0 ATPase of mitochondria, eliminating the oxygen consumption associated with oxidative phosphorylation [20]. For freshly isolated chondrocytes, oligomycin sensitive oxygen consumption accounted for 0.57 f mol $cell^{-1}$ h^{-1}. At day 7, the equivalent value was 55.72 f mol $cell^{-1}$ h^{-1}. Thus,