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Cardiac differentiation promotes mitochondria development and ameliorates oxidative capacity in H9c2 cardiomyoblasts

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ABSTRACT

H9c2 undergoing cardiac differentiation induced by all-*trans*-retinoic acid were investigated for mitochondria structural features together with the implied functional changes, as a model for the study of mitochondrial development in cardiogenic progenitor cells.
As the expression of cardiac markers became detectable, mitochondrial mass increased and mitochondrial morphology and ultrastructure changed. Reticular network organization developed and more bulky mitochondria with greater numbers of closely packed cristae and more electron-dense matrix were detected. Increased expression of PGC-1α proved the occurrence of mitochondrial biogenesis. Improvements in mitochondrial energetic competence were also documented, linked to better assembly between F_0 and F_1 sectors of the F_0F_1ATPsynthase enzyme complex.

1. Introduction

The energy demands of a cell change dramatically during the course of cell differentiation and development, then, in order to suit the current state of the cell, mitochondrial content and function are subject to a well-orchestrated regulation by both the nuclear and mitochondrial genomes (Hock & Kralli, 2009). Accordingly, mitochondrial biogenesis, namely the molecular control of mitochondrial turnover, content and number, is often included in cell differentiation programme, mainly when the differentiation lineage leads to phenotypes characterized by high energy demands, as is the case for skeletal and cardiac myocytes (Remels et al., 2010; Chen et al., 2010). Moreover, the regulatory control of mitochondrial mass, structure and function contributes to cardiac homeostasis and adaptation in response to physiological and pathological challenges, such as heart failure (Rimbaud et al., 2009), and is involved in cardiac ischemia/ischemic preconditioning (McLeod et al., 2005; Schwartz & Sack, 2008; Di Pancrazio et al., 2004; Lippe et al., 2009). More generally, the functional importance of mitochondrial biogenesis in maintaining cardiac homeostasis (Rimbaud et al., 2009; Ventura-Clapier et al., 2010) is reinforced by the finding that the genetic ablation of master regulators of mitochondrial biogenesis impairs the capacity of myocytes to adapt to different stress conditions (Schwartz & Sack, 2008).

Nowadays, many studies into cardiomyogenesis have focused on the cardiac lineage specification of differentiating stem cells (e.g., Chung et al., 2007; Chen et al., 2010) for which activation of mitochondrial biogenesis-associated genes has been documented (Chen et al., 2010). Generally, undifferentiated human embryonic stem cells (hESCs) have few mitochondria and they present immature ultrastructural features. As hESCs differentiate, the total number of mitochondria increases as does the number of mitochondria that exhibit a more mature morphology (Chen et al., 2010). Concomitantly, the rate of oxygen consumption and ATP production increase, whereas that of lactate production decreases (Chung et al, 2007). Notably, functional mitochondria in hESCs are necessary for cardiac differentiation, mitochondrial organization, sarcomerogenesis and contractile function (Chung et al., 2007). In summary, although this remains a promising and novel area of research, the data already indicate that the modulation of mitochondrial activity may present a useful tool for maintaining ESCs in a pluripotent state or for driving differentiation towards a specific lineage, in particular to the cardiomyocyte phenotype.

Mitochondrial biogenesis has also been demonstrated during liver development (Cuezva et al., 1997), skeletal muscle regeneration (Duguez et al., 2002; Shao et al., 2010), adipogenesis (Wilson-Fritch et al., 2003) and osteogenic differentiation (Chen et al., 2008). In particular, in skeletal myogenesis, mitochondrial biogenesis was reported to be fundamental, not only for the energetic metabolism of the cells, but also for the regulation of cellular proliferation and

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differentiation (Remels et al., 2010; Kraft et al., 2006). In fact, mitochondria are not only able to remodel their structure and function in order to meet their energetic needs better, but they are also able to directly participate in the process of differentiation. In accordance, the inhibition of biogenesis has been associated with restraining myoblast differentiation and with altering the capacity for mitochondrial activity to interfere with the ability of myogenic factors to induce terminal differentiation (Herzberg et al., 1993; Rochard et al., 2000).

The present study investigates an *in vitro* model of cardiomyogenic differentiation induced in H9c2 rat cardiomyoblasts by chronic treatment with all-*trans*-retinoic acid and reduced serum culture medium (Menard et al., 1999); the aim was to confirm that the cardiomyocyte phenotype is actually acquired in these cells and to ascertain whether mitochondrial biogenesis occurs in concomitance. We found mitochondrial mass to increase during H9c2 cardiac-like differentiation, as well as maturation-associated changes in mitochondria shape and structure. Mitochondrial biogenesis was confirmed by increased PGC-1α expression. Functional/structural promotion of oxidative phosphorylation was demonstrated to occur in concomitance. Specifically, a significant increase in phosphorylating respiration and maximal F_0F_1ATP synthase activity was shown, which appears to be connected to the increase in complex expression and assembly. Thus, given the crucial role of mitochondrial biogenesis and function in driving progenitor cell differentiation towards the cardiomyocyte phenotype, all-*trans*-retinoic acid-treatment of cultured H9c2 cells represents a useful model for studies aimed at understanding the mechanisms involved in mitochondrial biogenesis, cardiomyogenic differentiation and cardiac homeostasis.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

2.2. Cell culture and differentiation treatment

Rat cardiomyoblast-derived H9c2 cells (ATCC CRL1446), purchased from the American Type Culture Collection (Rockville, Md, USA), were grown and induced to differentiate toward the cardiac-like phenotype as described by Menard and collaborators (Menard et al., 1999). Following decreases in growth factor concentration, myoblasts rapidly lose their characteristics and become multinucleated myotubes; subculturing was therefore performed before cell confluence was obtained to prevent spontaneous trans-differentiation into the skeletal phenotype.

Differentiation was induced by culturing myoblasts in DMEM supplemented with 1% fetal calf serum and 10 nm all-*trans*-retinoic acid (RA) with the medium being replaced every 2 days. The effects of the differentiating treatment were monitored for at least 3 days and for a maximum of 14 days.

2.3. Confocal microscopy

5×10^4 cells were plated in complete medium on glass coverslips and grown overnight. Cells fixed with 3.7% paraformaldehyde in PBS were permeabilized with 0.2% Triton X-100 for 5 min and washed three times with PBS. Cells were then incubated overnight in blocking solution (1% bovine serum albumin in PBS) at room temperature with the following primary antibodies: anti-troponin 1 (cardiac isoform) mouse monoclonal antibody, (1:50) (Abcam, Cambridge, UK); anti-AIF rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); anti-cytochrome c mouse monoclonal antibody (1:200) (BD Biosciences Pharmigen, San Jose, CA, USA); rabbit polyclonal antibody raised against the β subunit of the F_1 portion of F_0F_1ATP synthase (1:100) (kindly provided by Prof. F. Dabbeni-Sala, University of Padua, Italy). After three washes with PBS, cells were stained for 2 h at room temperature with fluorescein-conjugated goat anti-mouse IgG (1:1000) or fluorescein-conjugated rabbit anti-goat IgG (1:200) (Chemicon International, Temecule, CA, USA). Following further washes, the coverslips were mounted in glycerol-based mounting fluid (Chemicon International) and examined using a laser scanning confocal microscope equipped with a 488–534 λ Ar laser and a 633 λ He–Ne laser (Leica TCS NT, Leica Mycrosystems, Wetzlar, Germany).

In some experiments, intact viable cells were incubated in complete medium with mitochondrion selective probes, i.e., 100 nM Mitotracker Red CMXRos (Molecular Probes, Eugene, OR, USA) or 10 μg/ml 5,5 ,6,6 -tetrachloro-1,1 3,3 -tetraethyl-benzimidazolecarbocyanide iodine (JC-1) (Molecular Probes) for 30 min at 37 °C. After staining and washing, cells were either observed directly, or fixed in paraformaldehyde and permeabilized in Triton X-100 as described above, before confocal microscopy examination.

2.4. Flow cytometry

Subconfluent cells were washed twice in PBS, trypsinized for 5 min and incubated with the mitochondrion selective probes, Mitotracker Red CMXRos 100 nM or with JC-1 10 μg/ml, for 10 min in complete medium at 37 °C in the dark. After washing, cells were suspended in PBS at 2×10^6 cells/ml and analyzed using a FACScan flow cytometer (Becton-Dickinson, New York, USA) equipped with a single 488 nm argon laser. Data were acquired in list mode and analyzed with cell Quest Software (Becton-Dickinson).

2.5. Transmission electron microscopy

Cell monolayers were (i) washed with fresh PBS; fixed with 2.5% glutaraldehyde (Agar Scientific Ltd, Stansted, Essex, UK) plus 2.5% formaldehyde (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) in 0.1 M PBS, pH 7.4, at 4 °C; (ii) washed with the same buffer; (iii) post-fixed with 2% OsO4 (Agar Scientific Ltd) dissolved in buffer as above, at 4 °C; (iv) dehydrated in graded ethanols; and (v) embedded in pure Araldite 502-Embed 812 Medium (Electron Microscopy Sciences).

Ultrathin sections were mounted on formvar-coated copper grids and stained using the conventional contrast agents uranyl acetate (2%) in ethanol solution (Electron Microscopy Sciences) and 1% lead citrate in aqueous solution (Carlo Erba, Milan, Italy). Observations and micrographic recordings were made using a Philips CM12/STEM transmission electron microscope (Philips, Eindhoven, Netherlands).

2.6. Western blot analysis

Cultured cells were harvested, lysed and separated by electrophoresis in 15% SDS/polyacrilamide gels under reducing conditions (Laemmli, 1970). Protein concentrations of loaded samples were determined according to the Lowry method (Lowry et al., 1951). Proteins were transferred onto nitrocellulose membranes pre-incubated at room temperature for 1–2 h with 5% non-fat dry milk in PBS containing 0.1% Tween 20 (PBS-Tween) by wet electroblotting in a buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. The membranes were blocked by incubation in PBS-Tween containing 5% non fat-dry milk and incubated overnight at 4 °C with the following primary antibodies: rabbit polyclonal antibody raised against the β subunit of F_0F_1ATP synthase (1:2000) (kindly provided by Prof. F. Dabbeni-Sala, University of Padua, Italy), anti-cytochrome c mouse monoclonal antibody (1:2000) (BD Biosciences Pharmigen), anti-transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-α (PGC-1α) rabbit polyclonal antibody (1:2000) (Abcam) and anti-β actin rabbit polyclonal antibody (1:3000) (Sigma). The washed membranes were then incubated with