Immune and Bioenergetic Profiling of a Human Dermal Fibroblast Cell Line

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The adult human dermal fibroblasts (HDFa) can be used in various research fields such as skin aging and wound healing, tissue regeneration and engineering as well as in cancer research. A better understanding of the mitochondrial physiology of HDFa cells is mandatory in order to further decipher the abnormalities related to pathophysiological conditions. The present study was aimed at providing a comprehensive characterization of the HDFa cell line with respect to their phenotype and bioenergetic profile. Flow cytometry revealed that HDFa cells express CD90, CD73, CD 29 and HLA-A2 markers, whereas in immune fluorescence they were positive for CD105, vimentin, and Ki-67 markers. The optimal parameters for mitochondrial respiration and glycolytic flux, respectively, were reported in experiments standardized for 30.000 cells/well.

Key words: HDFa cell line, flow-cytometry, immunofluorescence, mitochondria bioenergetics

Skin, the largest human organ of the body, has the major protective role as barrier against microbial invasion, toxic compounds and UV radiation, as well as all environmental factors that render the dermal layers susceptible to the aging process [1-4].

Fibroblasts exhibit a vital role in modulating skin physiology and pathology [5], being also able to communicate between them as well as with other skin cells [6]. Moreover, they are capable to release cytokines (e.g., interleukin 6) [7-9] and various growth factors via paracrine and autocrine interactions, thus participating in wound repair, inflammatory processes, immune responses, as well as in cell proliferation and apoptosis [5, 10, 11]. In the past decade, the role of fibroblasts has been evolved from a cellular mediator of skin aging to a source for tissue engineering strategies [12] view their remarkable phenotype plasticity [12]. Moreover, it has been recently discovered that dermal fibroblast of allogeneic origin do not possess an immune response, making them suitable for skin substitutes [13, 14].

Ultraviolet (UV) radiation is able to penetrate the epidermis and the dermis, leading to overproduction of reactive oxygen species (ROS) in the skin [1, 15] with the subsequent oxidative damage to cell membranes, lipids, mitochondria, and DNA [15]. UVB is considered the most hazardous environmental carcinogen [1, 16, 17] due to the deleterious consequences of overexposure, such as: sunburn, photoaging, immune suppression, and cancer [18, 19]. The present study was aimed at providing a comprehensive phenotypic and bioenergetic characterization of an adult human dermal fibroblast cell line that will be further studied in pathological settings (i.e., UVB exposure).

Experimental part

Material and methods

Cell Culture

Human dermal fibroblasts cell line (HDFa) were purchased from Cascade Biologics Invitrogen Cell Culture and were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 100U/mL Penicillin, 100 μg/ml Streptomycin and 20% Fetal Calf Serum (FCS). Cells were kept at 37°C in humid atmosphere containing 5% CO_2.

Immunofluorescence

HDFa cells were cultivated in 8-well plates. After cell adhesion, the medium was discarded, and cells were washed with phosphate-buffered saline (PBS), and fixed with methanol for 10 min at -20°C. When time elapsed, methanol was removed, and cells were washed again with PBS. Cells were further sequentially incubated with primary antibodies and fluorescein-conjugated secondary antibodies. The primary antibodies (Dako Cytomation, Glostrup, Denmark) used for staining were: Monoclonal Mouse Anti-Human Endoglin, CD105 (Clone SN6h);

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Polyclonal Rabbit Anti-Human, CD117; Monoclonal Mouse Anti-Human Smooth Muscle Actin/HRP (Clone 1A4); Monoclonal Mouse Anti-Human Cytokeratin, High Molecular Weight (Clone 34βE12); Monoclonal Mouse Anti-Human Ki-67 Antigen (Clone MIB-1); Monoclonal Mouse Anti-Vimentin (Clone V9); Monoclonal Mouse Anti-Human Caldesmon (Clone h-CD) and Monoclonal Mouse Anti-Human Smooth Muscle Myosin Heavy Chain (Clone SMMS-1). Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 594 (Molecular Probes, Invitrogen) were used as secondary antibodies for staining. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 10 minutes. The probes were analyzed by fluorescence microscopy using a Nikon Eclipse E800 microscope (Nikon, Tokio, Japan) equipped with fluorescence filters [20].

Flow cytometry study

HDFa cells were detached using 0.25% Trypsin-EDTA (Sigma), washed twice with PBS, and resuspended in 100 µL PBS at a concentration of 10^5 cells/ml. Cells were further incubated at room temperature, in the dark for 30 min with mouse anti-human fluorochrome-conjugated antibody at a dilution specified in the manufacturer's protocol. Cells were then washed twice with 1 ml Cell Wash Solution (BD Biosciences, San Jose, CA, USA) centrifuged for 7 min at 1500 RPM and then resuspended in 500 µL of the same solution for further analysis using the FACSCalibur flow cytometer (Becton-Dickinson).

Conjugated antibodies utilized included PE-conjugated CD117, IL-10R, CD109 (BD Pharmingen™), CD29, TGF-βR III (R&D Systems), as well as FITC-conjugated CD26, CD34, CD44, CD45, CD73, CD90, CD105, HLA-A2, HLA-DR (BD Pharmingen™). Some of the markers were located within the cytoplasm and required an additional permeabilization step, before intracellular immunofluorescence staining with monoclonal antibodies (BD Biosciences) according to manufacturer's instructions. Acquisition and data analyses were performed with the Flowing Software 2.5.1 [21].

Bioenergetic assay

The bioenergetic profile of HDFa cell line was assessed using the extracellular flux analyzer XF24 Flux Analyzer (Seahorse Biosciences) according to a previously described method [22, 23], with the simultaneous measurement of the oxygen consumption rate (OCR) as the indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR) as the indicator of the glycolytic conversion of glucose to lactate [24, 25]. Briefly, HDFa cells were seeded for 24 hours in Seahorse XF-24 cell culture plates at different densities: 10, 20, and 30 K cells/well, respectively. In the day of the experiment, DMEM was replaced by unbuffered XF assay medium (Seahorse Co.) supplemented with 25 mM glucose. Three sequentially injections were performed: 1 µg/mL oligomycin (an ATP synthase inhibitor), 3µM FCCP (carbonyl cyanide-4-(trifluoromethoxy)- phenylhydrazone, a classic uncoupler of oxidative phosphorylation), and 5 µM antimycin A (a complex III inhibitor), respectively. The following OCR parameters [25, 26] were analyzed and calculated:

- basal respiration - OCR measured before oligomycin injection;
- proton leak - the amount of O_2 used to maintain the proton gradient;
- ATP turnover - the percent of O_2 consumption devoted to ATP production, calculated as the difference between basal respiration and proton leak;
- maximal respiration - OCR measured after FCCP injection (the uncoupled respiration);
- reserve capacity - the difference between the maximal and basal respiration;
- non-mitochondrial respiration - the amount of O_2 consumption not due to mitochondrial processes;
- respiratory control ratio (RCR) - calculated as the ratio between the maximal respiration and the proton leak.

OCR was corrected for oxygen flux due to instrumental background and non-mitochondrial respiration, and it was expressed in units of nmoles/minute/nr of cells, while ECAR in m*p*H / min / nr of cells.

Reagents

All the other chemicals were from Sigma-Aldrich.

Results and discussions

Morphology of the HDFa Cell Line

In physiological conditions, the HDFa cells exhibited a fibroblast-like shape with intact membranes , as shown in figure 1.

Immunofluorescence of the HDFa Cell Line

Our analysis revealed that the HDFa cells were positive for 3 markers, namely Clone SN6h, Vimentin, and Ki-67 (fig. 2). CD105 Clone SN6h is a type I transmembrane protein, highly expressed on human vascular endothelial cells, being a endothelial cell marker associated with

Fig. 1. HDFa cells (Magnification 20x)

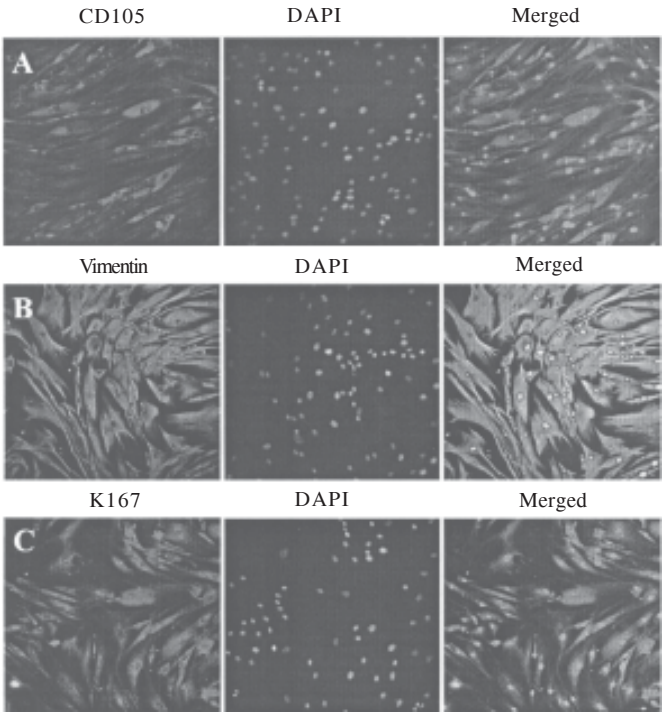

Fig. 2. Immunofluorescence microscopy of HDFa cell line. Cells were positive for CD105 (A), Vimentin (B), and Ki67 (C)

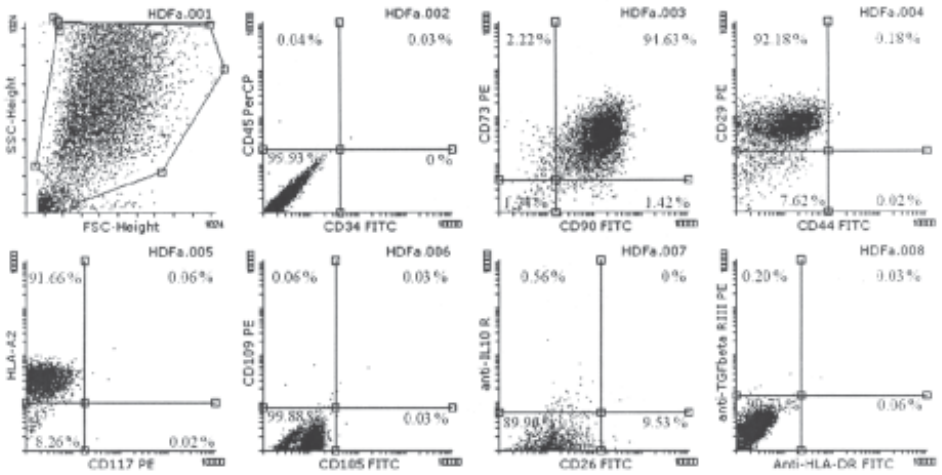

Fig. 3. Histograms of flow cytometric analysis of HDFa cells treated with different molecular markers.

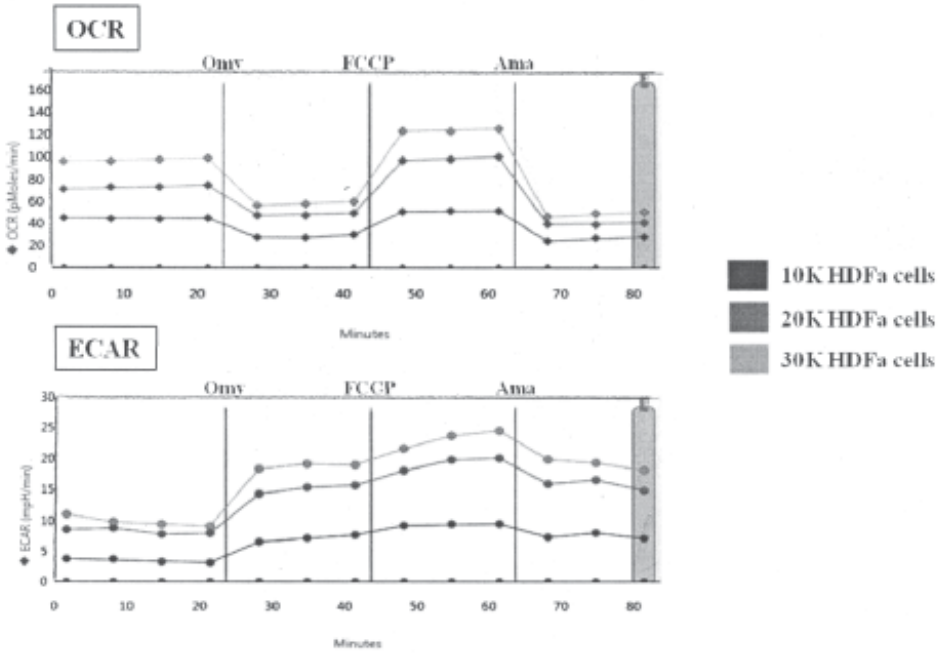

Fig. 4. Graphic representation of the OCR and ECAR recorded during the experimental protocol. Different numbers of cells were tested in order to obtain the optimal seeding density

Cell groups	OCR (nmoles/min/nr cells)						ECAR (mpH/min/nr cells)
	Basal respiration	Proton leak	ATP turnover	Maximal respiration	Reserve capacity	RCR	
10K HDFa	17	4	13	21	4	5.25	0.7×10^{-3}
20K HDFa	30	10	20	50	20	5	1.4×10^{-3}
30K HDFa	46	12	34	72	26	6	1.6×10^{-3}

Table 1
GROUPING MEASURED DATA: OCR AND ECAR PARAMETERS DATA ARE EXPRESSED AS MEAN VALUES (n = 6-8/GROUP)

proliferation [27]. Vimentin is an intermediate filament protein used to identify cells of mesenchymal origin [28], while Ki-67 is a protein expressed during all active phases of the cell cycle, used for the identification of proliferating cells in normal and neoplastic tissues [29].

Expression of Molecular Markers by the HDFa Cell Line

Flow-cytometry studies indicated that HDFa cells express CD29, CD73, CD90 and HLA-A2 markers, and are negative for CD34, CD45, CD44, CD117, CD105, CD109, CD26, IL-10R, HLA-DR and TGF-â RIII (fig. 3). CD 29 is a glycoprotein that acts as a fibronectin receptor and is involved in a variety of cell-cell and cell-matrix interactions, CD73 costimulates T cell activation, CD90 regulates hematopoiesis, cell growth and adhesion, and HLA-A2 plays an important role in cell-mediated immune responses and tumor surveillance [30, 31].

The experiments were aimed at standardizing the HDFa cell seeding density on OCR and ECAR values (fig. 4 and table 1).

As shown in figure 4, oligomycin was firstly injected, inducing a decrease in OCR due to the blockade of the intracellular ATP synthesis via OXPHOS; as a result, the energy production shifted to glycolysis, which lead to an increase in ECAR. FCCP was secondly injected and significantly increased OCR, as more O_2 was consumed to pump the excess protons back across the mitochondrial membrane, while ECAR increased due to cells attempt to

maintain their energy balance by using glycolysis to generate ATP. The injection of antimycin A induced a significant decrease of OCR, while ECAR was still maintained, since cells shifted to a glycolytic state, in order to maintain their energy balance (fig. 4).

We report a linear increase of OCR and ECAR as cell density increased (fig. 4), a relation that was also found when the OCR parameters and ECAR were expressed / number of cells (table 1).

The highest value of the respiratory capacity parameter (table 1) that reflects the cells' ability to respond to energy demand [32], was obtained for 30 K cells/well. The same result (table 1) was observed for RCR, a reliable indicator of mitochondrial respiratory function [32]. The ECAR data analysis showed that the highest value was obtained also for 30 K cells/well (table 1). Taken together, these data demonstrate that the optimal bioenergetic profile of HDFa cells was obtained at a density of 30 K cells/well; this value will be further used in order to assess the changes in the bioenergetic profile in pathological settings.

Conclusions

The present study was purported to provide a thorough immunophenotypic, bioenergetic and metabolic characterization of the HDFa cell line. Accordingly, we showed that the cells express CD90, CD73, CD 29, and HLA-A2 markers, and that 30,000 cells/well represents the appropriate density for future studies addressing mitochondrial (dys)function and toxicity.

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